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ENVIRONMENTAL BIOLOGY
STATE-OF-THE-ART SEMINAR

Patricia A. Archibald, Editor

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Organized by Environmental Protection Agency,
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Held at College of Saint Scholastica,
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Preface

By Direction of Congress, the Office of Research and Development, Environmental Protection Agency, implemented a new process for solicitation and evaluation of research grants in late 1979. An Office of Research Grants and Centers (ORGC) began operation in January, 1980. Research grants funded under the auspices of ORGC are not necessarily representative of the kinds of projects which the Agency funds as cooperative agreements and contracts through other programs at Headquarters and the regional research laboratories.

In April, 1980, the first annual generalized solicitation was issued. Initially, proposals were requested in four major areas, e.g., Environmental Engineering and Pollution Control Processes; Health Research; Environmental Chemistry and Physics; and Environmental Biology. Usually, eligibility is limited to universities and other non-profit institutions.

The program is extremely competitive with scientific merit of every proposal being evaluated by an extramural Peer Review Panel comprised of members nominated from the academic community, other agencies within the Federal government, and industry. A chairperson elected from the academic community administers the Peer Review Process. Only those proposals recommended for consideration for funding by the Peer Review Panel are considered further by ORGC. The final funding decision for each competitive research grant is made within the Agency relative to scientific merit, relevance to current Agency programs and availability of monies.

Dr. Freida Taub, of the University of Washington-Seattle, served as the first chairperson of the Environmental Biology Peer Review Panel. Her skills in organization and appreciation of detail have been important assets in the inauguration of the competitive grants problem in the area of Environmental Biology. Procedures developed by Dr. Taub will continue to be employed by subsequent chairpersons on both the Environmental Biology Panel and panels in the other fields of study.

Each Principal Investigator is required to present a seminar detailing his research. Al work presented at this seminar for Environmental Biology is by Principal Investigators who had their original proposals reviewed during Dr. Taub's term of office. The discussant for each paper, in most incidences, was one of the initial reviewers of the proposal as first submitted.

The Agency wishes to compliment both Dr. Taub and the Principal Investigators for the quality and depth of work presented.

STATE-OF-THE-ART SEMINAR
Environmental Biology

College of St. Scholastica
Duluth, Minnesota

July 20 - 22, 1981

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THE SENSITIVITY OF CRITICAL LIFE STAGES OF BENTHIC MEIOFAUNA TO DRILLING MUDS

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ABSTRACT

Static bioassays using benthic meiofauna (*Chromadorina germanica* [nematode], *Nitocra typica* [harpacticoid copepod], *Allogromia laticollaris* [foraminifera], and *Euplotes vannus* [ciliate]), as sensitivity indicators were run testing drilling muds from Mobile (Alabama) bay. Mud sample types were raw, desilter, and desander/desilter. Drilling mud mixtures with sand was an unfavorable substrate for several animals, restricting their body and feeding movements and causing them to starve to death. When the mixtures were embedded in agar to change their physical properties the animals moved and fed normally. The results of this preliminary study suggest that two of the animals tested, *Chromadorina germanica* and *Allogromia laticollaris* have the potential to be developed into bioassay tools for marine benthic muds.

INTRODUCTION

Microfauna (protozoa ≥ 50 μm) and meiofauna (nematodes, copepods, foraminifera, and similar sized [0.05-0.5 mm] small organisms) are among the most numerous group of marine animals and play key roles in benthic food webs (reviewed in 1). They consume and regulate benthic microfloral assemblages and they, in turn, serve as food for larger, more familiar animals. Because they live in intimate contact with the substrate as both epifauna and infauna, it has been suggested that they are among the most sensitive indicators of environmental quality (2, 3, 4). Nutritional and physiological ecological studies suggest that various larval (e.g. nauplei) stages are even more sensitive to unfavorable conditions than are adults (5).

Although there are indications that biocides such as pentachlorophenol and wetting agents such as barite adversely affect meiofaunal assemblages

(6, 7) little is known of the range of sensitivies of the group to such agents which are constituents of drilling muds. Since many species of micro- and meiofauna are easily cultivated in ordinary laboratory glassware under common laboratory conditions (8, 9) they have great potential as tools for the assay of benthic sediments which may have become impacted by various types of discharges.

MATERIALS AND METHODS

The species of meiofauna used in this study were isolated from the sublittoral zones of salt marsh tidal paines at either Towd Point on Peconic Bay, Southampton, Long Island, or Greater Sippewissett Marsh on Buzzards Bay, Falmouth, Mass. (8). The four species selected, Allogromia laticollaris (foraminifera), Nitocra typica (harpacticoid copepod), Chromadorina germanica (nematode), Euplotes vannus (ciliated protozoan) have worldwide distributions and are easily cultivated. Laboratory procedures for raising and manipulating the experimental animals followed those previously published (5, 9-15). Care was taken to make sure that inocula were uniform size or age. Ten Allogromia ~ 500 µm in diameter; 10 gravid female Nitocra; 30 Chromadorina from 15 day cultures; 100 log phase Euplotes served as inocula in experiments. The organisms were inoculated into experimental vessels (35 cm^2 disposable tissue culture flasks and 60 mm petri plates or 20 x 125 mm test tubes) along with excess algal food (1×10^6 cells/ml). The effects of the drilling muds on the algae used as food was tested separately. On the basis of a preliminary toxicity screen the following algae were selected from our collection as food for the animals: Navicula multica, Nitzschia ovalis, Fragilaria shiloi, and Cylindrotheca fusiformis. Incubation was at 25° C in an illuminated incubator (16 hr L/8 hr D cycle) (Scherer Cel 4-4).

The drilling muds from a Mobile, Ala. bay well were shipped frozen from the EPA Environmental Research Laboratory, Gulf Breeze, Florida. They were refrigerated (4° C) upon arrival and maintained at the same temperature for the duration of the study. Collection data, depth of sample, density, viscosity, pH, calcium content, water content, and diesel oil content were sent with the samples. A copy of the metal analysis and methodology used for the analyses (Al, Pb, Cr, Ba, Fe and Zn) were also transmitted to us.

Various muds were diluted and mixed very thoroughly with washed silica beach sand from the National Wildlife Refuge on Peconic Bay, Southampton, L.I., N.Y. (grain characteristics see 16). Sand was also the substrate for the controls. In the initial experiments with the 3 meiofaunal species, sand was diluted with drilling mud volumetrically 1:1, 2:1 and 4:1. Peconic Bay seawater was used as an overlay (15 ml in the 35 cm^2 flasks, 10 ml in the 60 x 15 mm petri dishes). Later tests with the algae were done at higher dilutions (to 1×10^{-6}). The effects of drilling muds on Euplotes vannus were studied in test tubes with the muds diluted with sand over the range of 10^{-2} to 10^{-6}.

In an attempt to separate the effects of surfactants in the muds from other potentially toxic materials drilling muds were mixed (v/v) with sterilized agar (10^{-1} to 10^{-6}) to create a solid suspension. As in the previous experiment the substrate was overlain with sea water.

The duration of the experiments varied with the organisms. *A. laticollaris* and *Chromadorina germanica* were observed weekly for 30 days. Experiments with *Nitocra typica* were observed weekly and terminated after 7 weeks. *Euplotes vannus* was observed after 7 and 14 days. The experiments were terminated by adding 10 ml of Rose Bengal/formalin to the test vessels. Data were collected separately from the liquid overlay and from the agar fraction. With the higher concentrations of muds the agar had to be broken up and resuspended to enable enumeration of the *Chromadorina germanica*. In lower concentrations quadrants were read and statistically compared.

All experiments were in quadruplicate. No additional food was added during the experiment. Evaporation of media was kept to a minimum with distilled water in the bottom of the large (150 mm) Pyrex dishes which held the smaller, 60 mm petri plates.

RESULTS

The initial dilutions of 1:1, 2:1 and 4:1 were too turbid to permit growth of the algae tested. Bacterial growth also contributed to the density. Concentrations above 1:100 permitted reproduction of *Navicula multica*, *Nitzschia ovalis*, *Fragilaria shiloi*, and *Cylindrotheca fusiformis*. Growth of the latter two species increased four-fold as the muds were successively diluted ($1{:}10^{-2}$ to $1{:}10^{-6}$) to the highest level tested; the growth of *N. ovalis* trebled over the same range and that of *N. multica* was unaffected. Growth of the algae in the 10^{-6} dilution was equal to that of the controls.

Of all the organisms tested, *Allogromia laticollaris* was the most sensitive to drilling muds. *Allogromia laticollaris* and *Chromadorina germanica* did not survive in the 1:1 or 2:1 dilutions of any of the mud samples tested; some were still viable after 14 days in some 4:1 dilutions. Specimens of both of these species were behaving abnormally in the 4:1 dilutions. However, many seemed to recover when transferred to fresh control media. Microscopic observations suggested that *A. laticollaris* were starving to death. Their color was pale and they did not extend any pseudopods. Some of the "near dead: had a dark brown, wrinkled appearance. Microscopic observations of *Chromadorina* gave the impression that the physical properties of the media were responsible for their toxicity. The movement of the animals was abnormal, they pulsed slowly instead of rapidly moving when agitated. Some viscous material adhered to many worms and their mouths were clogged with sediment particles. They were probably attempting to feed on dead algal material.

TABLE 1. THE EFFECT OF DRILLING MUDS DILUTED 1:10 ON THE VIABILITY AND REPRODUCTION OF MEIOFAUNA AND A CILIATED PROTOZOAN

		SHAKER 1-2	RAW 2-3	RAW 2-7	SHAKER 2-11	DESA/SI 2-20	RAW 3-2	DESILT 3-16	SHAKER 5-1
VIABILITY	*Chromadorina germanica*	++	+	+	++	+	+	++	+
	Euplotes vannus	+	+	+	++	+	+	+	+
	Allogromia laticollaris	+	0	+	+	0	+	+	0
	Nitocra typica	++	++	++	++	++	++	+	+
REPRODUCTION	*Chromadorina germanica*	+++	+++	++++	++++	+++	+++	+++	+++
	Euplotes vannus	+++	+++	+++	+++	+++	+++	+++	+++
	Allogromia laticollaris	+	0	+	+	0	+	+	0
	Nitocra typica	++++	++++	++++	++++	++++	++++	++++	++++

0 dead
+ variable viability
++ viability (100%)
+++ variable reproduction
++++ reproductive success (several generations)

TABLE 2. THE EFFECT OF VARIOUS DRILLING MUD FRACTIONS ON THE FECUNDITY OF AN HARPACTICOID COPEPOD, *NITOCRA TYPICA*

	CONTROL	1:1	2:1	4:1	10:1	20:1	50:1	100:1
RAW 2-7	308	401	390	386	390	389	363	354
S.D.	20.3	48.6	40.3	36.6	21.2	30.8	16.1	21.3
SHAKER 2-11	350	398	360	358	378	388	374	351
S.D.	14.6	31.4	28.6	20.6	23.6	16.4	11.3	7.6
DESA/DESI 2-20	381	361	360	319	366	371	369	363
S.D.	12.1	5.6	8.9	10.3	6.1	7.3	6.8	6.6
DESILTER 3-16	396	388	388	369	359	386	358	387
S.D.	6.9	8.1	5.3	6.3	7.3	4.1	3.2	5.3
RAW 3-2	323	431	351	352	361	370	381	372
S.D.	2.1	4.3	2.1	1.6	4.5	3.6	4.2	4.7
SHAKER 5-1	356	298	261	270	235	233	248	225
S.D.	9.1	4.6	2.1	3.4	2.9	3.0	2.6	2.8

Incubation 7 weeks, average of 4 replicates
S.D. = standard deviation

TABLE 3. THE EFFECT OF VARIOUS DRILLING MUD FRACTIONS ON THE FECUNDITY OF A MARINE BENTHIC CILIATE, *EUPLOTES VANNUS*

	CONTROL	$1:10^{-3}$	$1:10^{-4}$	$1:10^{-5}$	$1:10^{-6}$
RAW 2-7	187	167	250	99	175
S.D.	14.3	20.6	10.3	8.6	9.3
SHAKER 2-11	78	49	83	55	49
S.D.	18.6	13.6	14.9	15.3	12.1
DESA/DESI 2-20	57	84	68	66	48
S.D.	19.2	12.4	12.6	13.1	10.2
DESILTER 3-16	75	120	58	54	36
S.D.	13.9	10.3	12.1	11.6	12.4
RAW 3-2	89	171	119	104	38
S.D.	16.4	13.3	16.5	14.3	19.6
SHAKER 5-1	74	139	44	42	31
S.D.	18.4	12.1	9.3	8.6	11.1

Incubation 7 days, average of 4 replicates ; S.D. = standard deviation

TABLE 4. THE EFFECTS OF VARIOUS DRILLING MUD FRACTIONS ON THE FECUNDITY OF A MARINE BENTHIC NEMATODE, CHROMADORINA GERMANICA

DILUTED MUD SOLIDIFIED WITH 1.5% AGAR

	CONTROL	1:1	2:1	4:1	10:1	20:1	50:1	100:1
SHAKER 1-2	500	75	60	70	150	150	140	150
S.D.	5.5	4.3	3.1	2.8	2.9	3.3	3.6	3.8
RAW 2-7	480	162	158	300	404	390	388	400
S.D.	4.8	3.4	4.5	3.0	4.5	2.6	3.8	8.5
SHAKER 2-11	560	2168	2496	2032	1759	1800	1740	1896
S.D.	12.3	12.4	27.9	10.8	50.4	61.4	61.6	11.9
DESA/DESI 2-20	525	30*	30*	30*	30*	30	100	150
S.D.	4.6					0.0	1.5	2.5
DESILTER 3-16	601	40	40	36	38	36	40	180
S.D.	3.3	1.7	5.8	4.0	2.5	1.9	2.1	12.2
RAW 3-2	581	28	29	50	192	480	441	424
S.D.	5.8	3.2	3.8	7.1	13.8	29.8	17.7	8.4
SHAKER 5-1	567	39	45	46	28	26	40	200
S.D.	4.7	4.8	5.6	6.0	3.2	6.6	5.5	6.8

Incubated 30 days;average of 4 replicate cultures

* Dead S.D.= standard deviation

TABLE 5. THE EFFECTS OF VARIOUS DRILLING MUD FRACTIONS ON THE FECUNDITY OF A FORAMINIFERAN, ALLOGROMIA LATICOLLARIS

	CONTROL	$1:10^{-3}$	$1:10^{-4}$	$1:10^{-5}$	$1:10^{-6}$
RAW 2-7	300	40%s	40%s	40%s	40%s
SHAKER 2-11	280	10	10	175	350
DESA/DESI 2-20	320	dead	dead	dead	dead
DESILTER 3-16	319	100%s	100%s	100%s	100%s
RAW 3-2	319	90%s	80%s	89%s	90%s
SHAKER 5-1	301	90%s	80%s	90%s	80%s +

s = survival

+ = cytotomy and binary fission noted

Inoculum = 10 organisms; experiment terminated after 30 days

On the other hand, *Nitocra typica* were viable and reproduced in most muds (Tables 1 and 2). The animals gathered the algae in the inoculum into balls above the surfaces of the mud and spent most of their time in the balls or swimming between algal masses. Nauplei and copepodites also spent most all their time in the superficial algal masses. None were observed as infauna in the substrate muds. *Euplotes vannus* usually survived in dilutions of mud 1:10 or greater (Table 3). Some reproduction was noted at that dilution. The reproduction rate reached that of controls at dilutions above $1{:}10^{-3}$.

When some characteristics of the drilling mud fractions were altered by solidification in agar *Chromadorina germanica* survived at all the dilutions tested and reproduced in many (Table 4). In several muds, raw 2-7, shaker 2-11, and raw 3-2, reproduction rates in dilutions of 20:1 or greater approached those of controls. Reproduction exceeded controls in all dilutions of one mud sample (2-11). Inhibition of reproduction to less than half of controls was still noted in dilutions of 100:1 in 4 mud fractions (raw 2-7, desand/desilt 2-20, desilter 3-16, shaker 5-1).

Most drilling mud fractions were toxic to *Allogromia laticollaris* even when they were solidified with agar (Table 5). The animals reproduced only in the shaker mud 2-11. Control levels were reached in a $1{:}10^{-6}$ dilution of mud. The desand/desilted mud 2-20 was toxic at all concentrations tested. There was little change in the survival rate of animals in the remaining mud samples over a dilution range of $1{:}10^{-3}$ to $1{:}10^{-6}$.

DISCUSSION

There was a wide range in the sensitivities of the 4 species of meiofauna to the drilling mud fractions which were tested. The implication from this very limited study is that the drilling muds would greatly alter the meiofaunal assemblage structure but would not totally eliminate it. Those animals able to live on the walls of the vessels (e.g. *Euplotes vannus*) would most likely survive on aufwuchs and particles which settle on the surfaces of the mud. The growth of *Euplotes* in all the experimental and control vessels was rather poor compared to its reproductive potential (13). This animal is a bloom feeder. It does well on algal or bacterial food when the densities exceed 10^5 cells per ml. Below that level feeding rates are very slow. Since the algae in the initial inoculum did not reproduce at high concentrations of drilling mud fractions the animal quickly grazed the algae down to below optimum feeding levels. This result is in sharp contrast to some of our observations on New York harbor and estuary muds where bacterial counts in some samples were quite high. The ciliates in those test vessels quickly reached their carrying capacity in less than a week (Lee and Tietjen work in progress). Workers in Europe (Persoone, personal communication) are using this same ciliate for the assay of marine muds with heavy metallic lodes.

The survival of Nitocra was undoubtedly due to the large algal inoculum which provided refuge for the animal. It is problematical whether this is an artifact of test conditions or whether this and similar animals could survive under natural conditions since many harpacticoid copepods are highly selective feeders (5, 17). The assay protocol which we used needs re-examination if this animal is to be employed in the future.

Chromadorina germanica could be used as an assay organism using either of the 2 protocols tested. Previous studies have shown that molecules as small as acetate are not absorbed through the cuticle of marine nematodes. They enter only when the animal is feeding on small particles (15). If there are soluble toxic materials in many of the muds they are not getting into the animals. This would account for the survival noted in many tubes. Larval and juvenile stages with thinner cuticles failed to survive. The reproduction of the nematodes in the shaker 2-11 mud dilutions suggests that the test conditions were favorable for both the food organisms and the animals, otherwise the food would have been grazed down very rapidly.

Allogromia laticollaris was the most sensitive organism tested. It only reproduced well in a 1×10^{-6} dilution of shaker 2-11 muds. Survival was noted at a 10^{-3} dilution. One hundred percent survival was also noted in all the desilter 3-16 muds tested.

The progress to date on this project seems to indicate that both Chromadorina germanica and Allogromia laticollaris could be used as bioassay organisms for drilling muds. The results of this preliminary study suggests that reproductive rate may be a better criterion to observe than survival since the survivers are slowly starving to death. Much more time and effort need to be expended to make a bioassay using these 2 animals practical, but the potential is there.

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Comments on
The Sensitivity of Critical Life Stages of Benthic Meiofauna to Drilling Muds

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The grant, entitled "The Sensitivity of Critical Life Stages of Benthic Meiofauna to Drilling Muds," was recommended for funding because of the importance of these organisms in marine ecosystems, their sensitivity to pollutants, the expertise of the principal investigator to culture these organisms in the laboratory, and the possibility of developing bioassay techniques for testing benthic marine organisms. In addition, the proposed work was deemed timely because of the increasing use of drilling fluids for petroleum exploration and their possible effects on marine organisms and because of the widespread pollution of the littoral zone from other anthropogenic sources.

The meiofauna, which includes nematodes, copepods, foraminifera and other small organisms, consume detritus, bacteria and algae and form an especially important role in the marine food web. In addition, they are sensitive indicators of environmental quality in salt marshes, tide flats, estuaries and bays. Interactions between inorganic and organic wastes and between sediments are poorly understood and meiofauna species and abundance are the best indicators of environmental quality.

The principal investigator has done considerable work on the physiology, nutritional and ecological requirements of meiofauna. He has also successfully cultured many of these species in the laboratory and thus is well qualified to carry out this study. The principal investigator's expertise and facilities to test the toxicity of drilling fluids to meiofauna were important factors in the panel's decision to recommend funding.

The development of a bioassay procedure to test meiofauna individually is important for determining the relative toxicity of drilling fluids; however, the results should be used conservatively. Bioassays will give toxicity information about the drilling fluids and needs to be tested; but "drilling fluids" are not reagent grade and many different mixtures are used for drilling. Perhaps more important is the composition of the "muds" both produced from the drilling operations and those in the area which will be affected. These will, in time, be affected by interactions with pollutants already present. Bioassays will help elucidate the most sensitive organisms and may be used to separate effects from toxic substances and physical conditions such as suspended particulates and changes in the benthic substrates. However, the probable area impacted by drilling fluids, the duration of impact, and time for recovery can best be measured under field conditions in conjunction with laboratory experiments. For both laboratory and field studies drilling fluids and muds should be analyzed for heavy metals (such as Pb, Cr, Hg, Ag and Ba), appropriate organics, and physical properties which can be used as "tracers" or "markers."

INDICATORS OF CURRENT GROWTH RATE AS RAPID METHODS FOR TOXICITY TESTS WITH FISH LARVAE

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ABSTRACT

After a series of experiments conducted for the development of methodology and for providing an understanding of the physiological basis for the methodologies, a few tentative conclusions have been reached. Percentage glycine incorporation into protein by larval fathead minnow tissue (in vitro) is reflective of protein synthesis and provides an index of growth as evidenced by differences between fed and starved fish. Glycine uptake is highly correlated with total available glycine and is probably the result of active uptake and absorption. Glycine incorporation appears to follow Michaelis-Menten kinetics. The RNA:DNA ratio also provides an index of growth rate as evidenced by differences between fed and starved fish. Both percentage glycine incorporation and RNA:DNA ratio have been reduced at sublethal concentrations of Cr^{+VI} in preliminary tests and hopefully will provide a rapid indicator of the long term effect of toxicants on growth.

INTRODUCTION

In recent years two important goals of research in aquatic toxicology have been: 1) to find rapid techniques for the determination of the effect of toxic materials, and 2) to find methods for determining if concentrations of a toxicant which cause effects in laboratory assessments are the same concentrations which cause similar effects in the natural environment of the organism. Although exposure of a test organism to a toxicant over a full life cycle provides the most complete information on the effect of that toxicant and is the standard upon which the validity of other kinds of tests are based, full life cycle tests are expensive and time consuming.

Recent studies have developed techniques for more rapid determination of toxic effects on fish and other organisms by showing that with many toxicants, levels of no effect determined in full life cycle tests can be predicted by the more rapid method, e.g. the fish cough response (1). However, many rapid methods fail to be generally useful with a large number of toxicants, possibly because of their failure to measure a meaningful physiological process. McKim (2) and Macek and Sleight (3) have found that measurement of the toxic effect during a sensitive period in the life cycle of a fish, usually the embryonic and larval period, will provide an estimate of toxicity very similar to that determined by a complete life cycle test but these tests still require about 30 days. With the recently adopted federal Toxic Substances Control Act (TSCA), it is imperative that rapid indicators of chronic effects be developed to expedite the testing required on an exceedingly vast array of chemicals. These rapid tests must reduce the time and expense of the presently required embryo-larva tests, should measure a physiological process related to the fish's well-being, and if possible be useful in field applications as well as the laboratory testing. Two techniques are available that with modification may enable rapid measurement of the effects of a toxicant in both the laboratory and in unconfined field populations. These procedures are: 1) the amino acid incorporation growth index, and 2) the RNA-DNA ratio.

The ^{14}C-amino acid incorporation growth index was developed by Ottaway and Simkiss (4) as a relative measure of the rate at which a fish is growing at the time it is sampled. The original method consists of removal of scales from a live fish, incubation of the scales in ^{14}C-labelled glycine, then measurement of incorporation of the glycine. Close correlations have been shown between glycine incorporation and the rate of growth of different fish populations, different year classes, and fish in different seasons (4,5,6). In laboratory experiments where fish were stressed by factors that are known to influence growth rate, glycine incorporation was reduced by handling, oxygen deprivation, and starvation (6,7,8). The method is extremely sensitive to rapid changes in growth rate of the fish since circadian rhythms have been detected in both laboratory (7) and field studies (Adelman-unpublished data).

The RNA-DNA ratio is another potentially useful indicator of growth rate. Investigations of protein synthesis in various organisms have shown that ribonucleic acid (RNA) increases rapidly in growing cells whereas deoxyribonucleic acid (DNA) remains relatively constant (9,10,11). Various studies have used the RNA-DNA ratio to effectively demonstrate rapid changes in growth rate of fish due to changes in feeding regimes or environmental factors (e.g. 12,13,14,15). Buckley (16) found the RNA-DNA ratio to be a good index of growth rate in Atlantic cod (Gadus morhua) larvae.

Since these growth indicators respond rapidly to changes in growth rate and become an immediately measurable characteristic of the fish, they are potentially useful as rapid indicators of stress in both laboratory and field applications. Thus, the overall objective of the present study is to determine the effectiveness of the amino acid incorporation growth index and the RNA-DNA ratio as rapid methods for determination of the no effect level of toxicants. Specific objectives to be achieved in this evaluation are to: 1) determine if the amino acid incorporation and the RNA-DNA ratio methods will detect effects of toxicants on growth of fish in 96 hours or less, 2) develop methodology for amino acid incorporation by larval fish muscle tissue instead of scales in order to test the more sensitive life history stage 3) determine if further modification of the amino acid growth index by exposing the muscle tissue to both the toxicant and the amino acid in-vitro will also serve as a rapid indicator of effect on growth.

The following information is a summary of results of approximately the first 6 months of investigation and is largely development of methodology.

AMINO ACID INCORPORATION

DEVELOPMENT AND VERIFICATION OF PROCEDURES

General Methodology

The methodology for measurement of the amino acid incorporation index of current growth rate has previously been accomplished only on older fish by using scales. One or more scales are removed from the fish and quickly immersed in a physiological saline containing ^{14}C-labelled glycine. After incubation in the glycine the scales are rinsed, the tissue is digested, and radioactivity that has been taken up by the scale is counted by liquid scintillation. Final uptake data are reported as concentration of glycine per some measurement of size such as scale weight or area in order to normalize results among different sized scales. Glycine was chosen since it is the amino acid of highest concentration in scale proteins.

Since part of the objective of the present study is to use incorporation of an amino acid as an indicator of growth rate in larval fish rather than older fish with scales, considerable development of methodology has been required. It was assumed that the epaxial musculature including the dermal and epidermal tissue and attached fins would be the only practical tissue to incubate in the amino acid. Experiments have thus been directed at determining if these tissues would take up the radiolabelled amino acid and if uptake would be related to growth rate. Although glycine is not the most common amino acid in muscle protein, it is one of the more common ones. To maintain continuity with studies on uptake by scales, ^{14}C-labelled glycine was chosen for the larval tissue.

The larval tissue for most all of the completed experiments was prepared by placing a fathead minnow (Pimephales promelas) larva on an iced glass plate and cutting it transversely with a razor blade just posterior to the gas bladder. The posterior section of the larva was then immediately placed in the incubation medium of a physiological saline and ^{14}C-labelled glycine and gently agitated in a 25C water bath during the incubation period. After incubation a variety of procedures have been attempted during the procedural development.

Total Glycine Uptake

An initial series of experiments was conducted that ultimately produced negative results and led to a different approach. Three experiments were first conducted to: 1) determine the relationship of glycine uptake by the larval sections to time of incubation, 2) to determine if a prolonged rinse was necessary after incubation and 3) to determine which of three buffer systems would result in the greatest and most linear uptake with time. The prolonged rinse was not necessary; of the three buffers tried, two zwiterionic buffers and one bicarbonate buffer, the latter produced the highest and most linear uptake and was the least expensive, although pH changes were the greatest. Uptake was approximately linear with time from 0.5 to 4 hours.

At this point an experiment was conducted to determine if a protein synthesis inhibitor (cycloheximide) would diminish uptake of glycine by the fathead larval sections. The cycloheximide had no effect at any of four concentrations, thus at least most of the glycine uptake that was being measured was not incorporated into newly synthesized protein. Finally, an experiment was conducted to compare glycine uptake in sections taken from fathead larvae that had been feeding with those that had been starved for 2 days. When the size differences between the faster growing, fed fish and the slower growing, starved fish were normalized by surface area, there was no difference in glycine uptake. Thus, the procedures used up to this time would not allow the use of glycine uptake as an index of growth.

Glycine Incorporation Into Protein

Two new techniques were next considered as possibilities for development of the procedure. Since uptake of glycine is an active process that has been shown at least in rainbow trout intestinal cells to be a function of glycogen content (17), an experiment was conducted to determine if the presence of glucose along with glycine in the incubation medium would enhance uptake and differentiate between fast growing and slow growing fish. In addition, general methodology was altered so that glycine actually incorporated into protein would be measured and differentiated from glycine in the free amino acid pool. The following procedure was used:

> Fathead minnow larvae (140) were divided between two tanks; one group was fed brine shrimp and the other starved for 48 hours. Tissue sections were incubated for 2 hours, 15 per vial, in physiological saline which contained either glycine (0.4 μCi/ml only or glycine and glucose (10mM), and each of those with (100 μg/ml) or without cycloheximide. After incubation and rinsing the larval sections were homogenized in 2 ml of distilled water. One hundred μl of this homogenate was digested and radioactivity counted to measure total uptake of glycine. Protein was precipitated from a 500 μl sample of the homogenate by the addition of cold (1.5 ml) 8% trichloroacetic acid (TCA) and centrifuged for 10 minutes at 10,000 rpm ($4^{o}C$). The protein pellet was then washed and centrifuged a second time with cold 6% TCA. This pellet was digested and radioactivity counted to determine glycine incorporated into protein. A second protein pellet was prepared from 500 ul of the homogenate for determination of total protein by a modified Lowry method (18).

Glucose had no apparent effect on total uptake of ^{14}C-glycine and in fact may be competitive with glycine (19) (Table 1).

TABLE 1. EFFECT OF GLUCOSE AND CYCLOHEXIMIDE ON TOTAL UPTAKE AND INCROPORATION OF ^{14}C GLYCINE INTO PROTEIN (pmol/μg PROTEIN) BY FED AND STARVED FATHEAD MINNOW TISSUE SECTIONS

Feeding Regime	Cyclo-heximide*	Glucose	Total Glycine	Incor-porated Glycine	% Incor-porated	Inhibition by Cyclo-heximide (%)
			Experiment 1			
Fed	No	No	0.192	0.0088	4.58	-
Fed	Yes	No	0.235	0.0019	0.79	82.8
Fed	No	Yes	0.206	0.0094	4.58	-
Fed	Yes	Yes	0.263	0.0023	0.86	81.2
Starve	No	No	0.360	0.0148	4.12	-
Starve	Yes	No	0.559	0.0068	1.12	72.8
Starve	No	Yes	0.541	0.0031	0.57	-
Starve	Yes	Yes	0.342	0.0038	1.12	?
			Experiment 2			
Fed	No	No	0.219	0.0114	7.11	-
Fed	Yes	No	0.183	0.0069	3.78	46.8
Fed	No	Yes	0.252	0.0156	6.19	-
Fed	Yes	Yes	0.219	0.0056	2.58	58.4
Starve	No	No	0.236	0.0070	2.99	-
Starve	Yes	No	0.219	0.0070	3.39	?
Starve	No	Yes	0.286	0.0109	3.81	-
Starve	Yes	Yes	0.247	0.0061	2.47	35.1

* 100 μg/ml in Experiment 1 and 1000 μg/ml in Experiment 2.

Cycloheximide inhibited glycine incorporation in both fed and starved fish by about 70-80%. Except for the starved-glucose treatment, cycloheximide seemed to enhance total uptake of glycine. The results from the starved-glucose treatment were inconsistent with the other groups and are presumed to be in error. It is apparent that total uptake masks the small amount of ^{14}C-glycine incorporated into protein. The starved fish generally had a greater total uptake and incorporation of glycine after adjustment by protein content, but the percentage incorporation seemed reduced.

A repeat of the previous experiment was conducted except that the concentration of cycloheximide was increased 10X to 1000 μg/ml. Total uptake across all groups was more uniform and again glucose had no effect (Table 1). The increased cycloheximide was actually less effective at inhibiting protein synthesis and also less effective among the starved than the fed fish. The percentage incorporation was again less among the starved fish indicating that percentage incorporation would provide an index of growth rate.

Relationship of Glycine Concentration to Incorporation

The next two experiments were directed at determining the relationship between concentration of glycine in the incubation medium and both total uptake and incorporation. In order to minimize expenses for the large amount of ^{14}C-glycine that might be needed, it was assumed that the tissue sections would take up and incorporate either ^{14}C-labelled (hot) or unlabelled (cold) glycine without preference, i.e. in proportion to their concentration in solution. Thus, the higher concentrations of glycine needed in these experiments were prepared by mixing known quantitites of hot and cold glycine and adjusting the measured radioactivity taken up or incorporated by the proportion of hot glycine in the mixture. The experimental procedures were as follows:

> Larval fathead tissue sections were incubated for 2 hours in vials containing six different concentrations of total glycine (15 tissue sections per vial, 2 vials per concentration). Vials were then placed in an ice water bath and homogenization, protein precipitation, protein concentration determination, and glycine uptake and incorporation determination proceeded as previously described. Glycine concentration ranged from 9 to 1800 μM concentration. The lowest percentage of radioactive glycine was 8% in the 1800 μM concentration. In the 9 to 44 μM glycine concentration, 100% was radiolabelled.

Total uptake was linearly related to concentration of glycine ($p < .01$), with a regression equation of

$$A = 736 + 14.86\ U \quad (R^2 = 99.3\%)$$

where A is the concentration of glycine in the incubation medium and U is the total glycine uptake. Incorporated glycine was also linearly related to concentration ($p < .01$)

$$A = 241 + 1.64\ I \quad (R^2 = 94.4\%)$$

where I is glycine incorporated into protein. Percentage of total uptake incorporated into protein was erratic in relation to concentration, but seemed to decline at higher concentration (Table 2).

The radioactive glycine used in these studies is purchased in a hydrochloric acid (HCl) solution. After the previous experiment was completed, it was realized that the pH of the incubation media containing the higher glycine concentrations had been lowered by the relatively large addition of HCl. The experiment was repeated with additional concentrations of glycine and with the HCl neutralized with equivalent normality NaOH and the Na and K in the physiological saline increased to counteract dilution.

As in the previous experiment, total glycine uptake was again linearly related to concentration ($p < 0.01$),

$$A = 1510 + 30.64\ U \quad (R^2 = 97.7\%)$$

Total uptake was considerably higher than in the previous experiment at most concentrations of glycine (Table 2). The reason for this is not known and seems unlikely to be related to the control of pH since both relationships were linear. Glycine incorporated into protein reached a plateau with glycine concentrations in the incubation media above 600 µM (Fig. 1).

TABLE 2. MEAN TOTAL UPTAKE AND INCORPORATION OF GLYCINE IN RELATION TO GLYCINE CONCENTRATION IN THE INCUBATION MEDIUM

Glycine concentration (µM)	Total glycine (pmol/µg)		Incorporated glycine (pmol/µg)		% Incorporated	
	Exp. 3	Exp. 4	Exp. 3	Exp. 4	Exp. 3	Exp. 4
9	0.348	0.392	0.062	0.053	17.8	13.6
18	-	0.711	-	0.083	-	11.6
36	0.732	1.173	0.109	0.173	14.9	14.7
72	-	3.364	-	0.402	-	12.0
144	2.677	6.241	0.561	0.476	21.0	7.6
300	-	11.148	-	0.739	-	6.6
600	11.500	27.733	1.775	1.110	15.4	4.0
900	-	27.120	-	1.377	-	5.1
1200	18.550	39.228	2.017	1.354	10.9	3.5
1500	-	42.971	-	1.344	-	3.1
1800	26.900	58.120	3.138	2.003	11.7	3.4

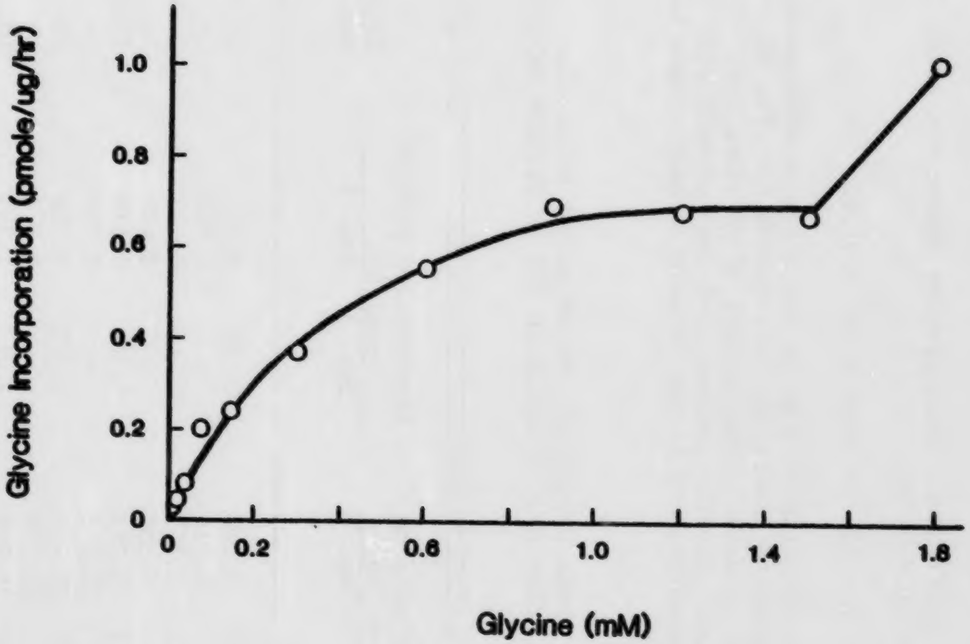

Figure 1. Incorporation of glycine into protein in relation to glycine concentration in the incubation medium.

The increased incorporation at 1800 μM may indicate the complex kinetics of different enzyme variants involved in the protein synthetic processes. A Lineweaver-Burk reciprocal plot ($R^2 = 0.98$, 9d.f.) resulted in a maximum velocity (Vmax) of 0.665 pmol glycine per μg protein per hour. The differences in actual glycine incorporation and percentage incorporation between the two experiments were probably due to the neutralization of the glycine in the latter and those results are considered more representative of the actual relationships. (Table 2).

Relationship of Glycine Incorporation to Time

Since it had now been established that glycine incorporation rather than uptake would be used as a growth index, it was necessary to reexamine the relationship with time. An experiment was conducted with fed and starved (24 hour) fathead larvae sampled from the incubation media at 15 minute intervals for 2 hours. Glycine concentration in the incubation medium was 290 μM with 4% radiolabelled.

Glycine incorporation increased with time, more or less linearly through 105 minutes, then with a sudden increase during the last 15 minutes (Table 3, Fig. 2). At this time no explanation has been found for that increase. Percentage glycine incorporated for both fed and starved fish generally increased with time until incorporation became asymptotic at about 90 minutes. The reduction in percentage incorporation among starved fish is reflective of their reduced growth rate. Tissue sections should be incubated for at least 90 minutes to allow the rate of incorporation of glycine to equilibrate with the rate of uptake and become constant with time.

TABLE 3. TOTAL GLYCINE UPTAKE (pmol/μg protein), GLYCINE INCORPORATION INTO PROTEIN (pmol/μg protein), AND PERCENTAGE INCORPORATION AT VARIOUS TIME INTERVALS FOR FED AND STARVED FATHEAD MINNOW LARVAE

Minutes of Incubation	Total glycine		Incorporated glycine		% Incorporated	
	fed	starved	fed	starved	fed	starved
15	20.571	17.905	0.524	0.486	2.5	2.7
30	19.619	14.667	0.848	0.819	4.3	5.6
45	17.904	29.333	1.381	1.286	7.7	4.4
60	20.571	21.714	1.990	1.695	9.7	7.8
75	32.380	31.238	2.667	2.267	8.2	7.3
90	26.875	26.875	3.286	2.452	12.2	9.1
105	27.428	31.048	3.524	2.857	12.8	9.2
120	47.238	52.000	5.571	4.952	11.8	9.5

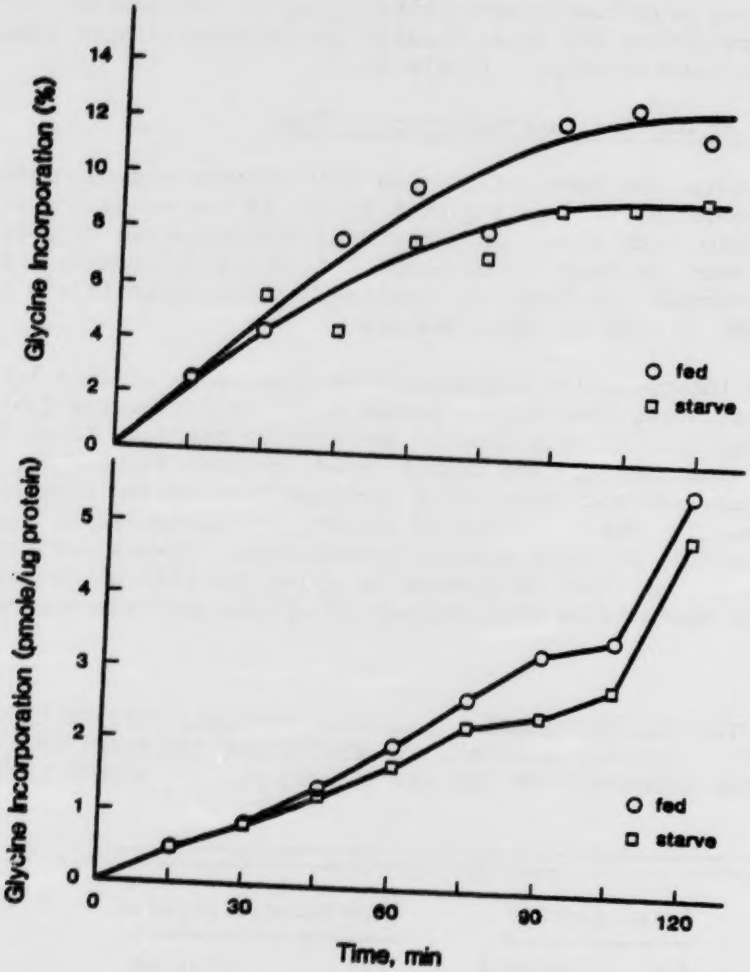

Figure 2. Glycine incorporation (bottom) and percentage glycine incorporation (top) into protein in relation to time for fed and starved larvae.

RNA-DNA STUDIES

METHODOLOGY VERIFICATION

Chemical Analysis

Studies of RNA, DNA, and their ratio have been pursued in order to determine if these measurements would also provide a rapid index of the effect of a toxicant on growth rate of larval fish and to determine if this methodology would be more sensitive than amino acid incorporation.

RNA and DNA have been analyzed in homogenized larval fathead minnows by the Schmidt-Thannhauser method (20), modified to measure microgram quantities of nucleic acids. RNA is measured by ultra violet (UV) absorbance (260 nm) after alkaline hydrolysis. DNA is also measured by UV absorbance (268 nm) after separation from protein by hot acid extraction (16). Protein remaining after RNA and DNA removal is measured by the Lowry method (18). After an experimental treatment the above analytical procedures have been performed either immediately on fresh larvae or on larvae that had been frozen in liquid nitrogen and stored at -80C until analysis.

Relationship to Growth Rate

An initial experiment was conducted to determine differences in RNA, DNA, and their ratio on fed and starved fathead minnow larvae. Two groups of larvae were fed or starved for 33 hours to create a growth rate difference. Two duplicate samples of 10 fish from each treatment were analyzed for RNA and DNA.

There were substantial differences in RNA, DNA, and their ratio between the fed and starved groups (Table 4).

TABLE 4. RNA, DNA (μg/larva) AND RNA:DNA RATIO FOR GROUPS OF FED AND STARVED FATHEAD MINNOW LARVAE

Measurement	FED		STARVED	
	Sample 1	Sample 2	Sample 1	Sample 2
RNA	11.66	12.72	1.59	2.12
DNA	6.40	6.80	2.72	2.80
RNA:DNA	1.82	1.87	0.58	0.76

The actual quantities of RNA and DNA are based on amount per larva and since the fed and starved fish differed in size, direct comparison cannot be made. Later tests have measured protein present in each sample so that size differences as reflected by protein differences can be normalized. However, in the present test the RNA:DNA ratio is a valid comparison because DNA is reflective of the number of nuclei present and thus theoretically an index of size.

An experiment was next conducted to determine the effect of fish age and size on the RNA:DNA ratio. Fish were sampled on days 1, 4, 8 and 12 for RNA, DNA, and protein analysis. Due to increasingly large within sample size variance with age, no statistically significant results were obtained for RNA:DNA ratio. However, it appeared as if neither size nor age had an independent effect on the ratio and thus the ratio for fish up to 12 days was not dependent on size (Table 5). DNA content was highly correlated with protein ($r = .992$, d.f = 10) and thus reflective of size.

TABLE 5. EFFECT OF SIZE AND AGE ON THE RNA:DNA RATIO DURING A 12-DAY PERIOD

Day	Mean dry Weight of larvae (mg)*	Mean Protein (µg)	RNA (µg/mg protein)	RNA:DNA
1	0.113	42.15	130.7	2.00
4	0.354	72.00	140.7	1.89
8	0.598	156.05	124.6	2.08
12	1.217	499.93	114.5	1.95

*Determined from a different subsample of fish.

Two experiments were conducted to determine if density of larvae in experimental chambers to be used for toxicity tests had an effect on the RNA:DNA ratio. Results were inconclusive although there was a slight indication that more than 30 larvae resulted in decreased ratios.

TOXICITY TEST OF HEXAVALENT CHROMIUM

Two preliminary toxicity tests of hexavalent chromium (Cr^{+VI}) were conducted and a 30-day larval test is underway. In the first preliminary test, 30 1-day old fathead minnow larvae were randomly assigned to each of six Cr^{+VI} concentrations and a control in a flow-through diluter modified from Mount and Brungs (21). After 96 hours groups of fish were taken from each treatment for the RNA,DNA analyses, and glycine incorporation exposures. There was no clear indication of an effect of Cr^{+VI} on any growth index although the lowest percentage incorporation did occur at the highest Cr^{+VI} concentration as did the lowest RNA:DNA ratio except for the lowest treatment where there may have been a procedural error in subsampling (Table 6).

TABLE 6. EFFECT OF Cr^{+VI} ON RNA:DNA RATIOS AND GLYCINE INCORPORATION IN FATHEAD MINNOW LARVAE

Cr^{+VI} (μg/1)	RNA:DNA	Glycine uptake (pmol)	Glycine Incorporation (pmol)	% Incorporation
0	2.04	492	31.4	6.4
11	1.76	423	24.8	5.9
42	2.03	489	24.4	5.0
63	2.07	508	39.8	7.8
135	2.02	518	30.9	6.0
230	2.04	519	32.4	6.2
440	1.93	465	22.9	4.9

Unless fish were more sensitive in this test than the one discussed below, the low ratio and glycine incorporation in the high treatment were probably an artifact.

In a second experiment with hexavalent chromium, two series of Cr^{+VI} concentrations, increased from the previous test, were run in duplicate. The test chambers in one series contained 15 larvae which were sampled at 96 hours for RNA:DNA ratio. The chambers in the other series contained 35 larvae which were sampled at 120 hours for RNA:DNA ratio and glycine incorporation.

The increased Cr^{+VI} concentrations resulted in a visually observable effect on growth and some mortality occurred in the higher treatments. The RNA:DNA ratios were clearly affected at both 4 and 5 days at Cr^{+VI} concentrations between 2.4 and 4.1 mg/1 (Table 7). Differences between 4 and 5 days may be due to density. The percentage incorporation of glycine seemed to be affected between the next higher treatment groups, i.e. 4.1 and 7.6 mg Cr^{+VI}/1 (Table 7).

With these results, a full length (30 day) embryo-larval test was undertaken to examine sensitivity of the RNA:DNA ratio at 4 days for predicting the effect of Cr^{+VI} on growth over 30 days. Glycine incorporation trials will also be conducted but results of these tests are not yet completed.

CONCLUSIONS AND FUTURE STUDIES

GLYCINE INCORPORATION

The incorporation of glycine as measured in TCA extracted larval tissue homogenates is due to protein synthesis as evidenced by cycloheximide

TABLE 7. EFFECT OF Cr^{+VI} ON RNA:DNA RATIOS AND GLYCINE INCORPORATION IN FATHEAD MINNOW LARVAE

Cr^{+VI} (mg/l)	RNA:DNA		Glycine Uptake (pmol)	Glycine Incorporation (pmol)	% Incorporation
	4 days	5 days			
0	2.72	2.00	1013	82.2	8.1
1.2	2.51	2.07	934	78.7	8.5
2.4	2.31	2.12	1134	92.9	8.2
4.1	2.08	1.60	812	75.7	9.3
7.6	1.57	-	581	42.9	7.4
12.8	1.20	-	544	35.4	6.5
21.0	-	-	514	33.3	6.5

inhibition of incorporation. Total uptake is dependent on size of the exposed tissue and may be the result of both active uptake and physical absorption; thus, total uptake is not necessarily reflective of glycine incorporation. For example, starved fish may have equal or greater uptake on a per size basis when compared to fed fish, but their percentage incorporation of glycine is less. Because a measure of protein synthesis should be a better index of growth rate than combinations of active uptake or absorption, the analysis of glycine incorporation as indicative of protein synthesis will provide a better index of growth rate than total uptake. In order to normalize results for different sized fish, the dimensionless unit of percentage incorporation should provide an index unbaised by size. An additional experiment using groups of different sized fish will be conducted to verify this assumption.

Glycine uptake is highly correlated with the total available glycine in the incubation medium but incorporation is not directly proportional to available glycine. Rather, incorporation appears to follow Michaelis-Menten kinetics.

Results of the preliminary toxicity tests with Cr^{+VI} appear promising and additional toxicity testing will be conducted over the next year at the University of Minnesota laboratory and in conjunction with ongoing projects at the Environmental Research Laboratory-Duluth. There will be a continued effort devoted to determination of the biological basis for the observed experimental results. Autoradiography of larval tissue sections will be used to examine the location of the protein synthetic activity and improvement in overall methodology will be sought in an attempt to understand and reduce the cause of sample variability.

RNA:DNA RATIO

The major problem still hindering the RNA:DNA ratio experiments is analytical variability. Continued effort will be devoted to improvements including measurements of analytical precision and efficiency of extraction of the proteins and nucleic acids. Also, the experiment to establish the size independence of percentage glycine incorporation will be used for establishment of size independence of RNA:DNA. As with the glycine incorporation methodology, the same toxicity tests will be used to compare RNA:DNA ratios with toxicant effect on growth and to compare the sensitivity of the two indices.

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Comments on Indicators of Current Growth RAte As Rapid Methods for Toxicity Tests with Fish Larvae

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One of the aims of environmental toxicology is the development of rapid, simple, and inexpensive assays that will reflect accurately the physiological effects of a toxicant on the biota in situ. Many such assays exist, using test organisms ranging from microbes to animals and in time required from hours to complete life cycles of months. Assays using whole animals and life cycles seldom meet the above criteria, although ultimately all assays must be correlated with complete life cycle evaluations, and assays using indicator organisms do not always reflect the more complex physiological responses of the higher organisms of concern in situ.

The objectives of this project are to develop and test two rapid methods to determine the "no effect level of toxicants" at the molecular level. One method involves measuring the uptake and incorporation into protein of a ^{14}C-labeled amino acid (glycine), and the other method involves determination of RNA:DNA ratios. Both methods are sensitive indices of cellular activity and, therefore, should quickly and efficiently detect the toxicity of a spectrum of potential toxicants. The technique involves the use of fat head minnow larvae tissue (the entire portion of the larva posterior to the gas bladder), and therefore, it uses a test organisms that is physiologically similar to a major group of higher organism of concern in aquatic environments. An important advantage of this assay is that it requires less than 96 hours to conduct in contrast to the 30 days usually required for estimating toxicity during the embryonic and larval period of fish. Another advantage is that a certain amount of redundancy is built into the assay: i.e., amino acid incorporation and RNA synthesis, in essence, measure the same phenomenon, protein synthesis. This redundancy, however, may provide some bonus information on cellular mechanisms of pollutant toxicity, especially if amino acid incorporation and RNA synthesis are not correlated. For example, the uptake of the amino acid may be subject to catabolite repression and other changes in metabolism (e.g., in active uptake and permeation), whereas RNA synthesis may be unaffected, thereby indicating the cellular level at which the toxicant is operating.

The Principal Investigator is methodically developing and verifying the procedures, as suggested by the original review panel, and has made reasonable progress since the grant was awarded. Additional testing is obviously needed to resolve a number of questions (e.g., use of RNase and DNase to verify the RNA:DNA ratios now determined by absorbance after alkaline and acid treatments, respectively; the influence of the shock of beheading on uptake and incorporation of the labeled amino acid). The major need, however, is to increase the number of larvae per test and the number of tests so that reliable statistical analyses can be applied to the data.

In summary, the review panel recommended support of this project, as it concluded that the proposal was scientifically sound, that the Principal Investigator was competent to do the work, and that the data obtained would have high utility to EPA, especially as some cooperative studies with EPA-Duluth and the Chesapeake Bay Institute are planned.

GROWTH KINETICS OF AEROMONAS HYDROPHILA IN FRESH WATERS AND THEIR RELATIONSHIP TO TROPHIC STATE

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ABSTRACT

A previously reported study, showing that the *in situ* *Aeromonas hydrophila* densities in bodies of fresh water are correlated with their trophic states, was extended to examine the potential of the water to support the growth of seeded *A. hydrophila*. No growth of the seeded bacteria occurred in untreated (raw) water samples incubated at room temperature irrespective of the trophic state of the water body or the season when the samples were collected. Multiplication to relatively high densities was observed, however, in filtered-autoclaved water samples. Moreover, the maximum *A. hydrophila* densities attained greatly exceeded the summer *in situ* densities for the same Rhode Island ponds.

The generation times of the organism, determined for the ten Rhode Island ponds utilized in the growth studies, were strongly correlated with the Relative Trophic Index (RTI), a measure of trophic state. Of those parameters comprising this index, total phosphorus, chlorophyll *a* and Secchi depth correlated strongly with the generation time for the organism. Water conductivity was also correlated with generation time. Maximum *A. hydrophila* densities observed in the filtered-autoclaved water samples were not correlated with any individual parameter, the RTI, or generation time.

INTRODUCTION

The authors, in an earlier publication (1), reported that the *in situ* densities of an autochthonous, heterotrophic bacterium, *Aeromonas hydrophila*, were inversely correlated with the Relative Trophic Index, RTI (2), of fresh water environments. These findings suggested that this bacterial parameter could be useful in trophic state assessments and possibly in the early prediction of long term limnetic changes in these aquatic environments. However, the reasons for the observed correlation between the densities of *A. hydrophila* and the trophic state of fresh waters have yet to be determined.

A number of factors could directly or indirectly influence the A. hydrophila densities attained in limnetic waters. Included are nutrient availability; physical parameters such as temperature, pH, dissolved oxygen, suspended solids, etc.; antibiosis from and competition with other biota; and predation by protozoa, bacteria, or bacteriophage. Of these, only the effects of temperature on the in situ densities of A. hydrophila in natural waters (1) and the physiological activities of the organism in culture (3) have been well documented. Some data are available, however, on the growth kinetics of the organism in nutrient supplemented tap waters (4). These data indicate that the generation time is substantially influenced by added sources of carbohydrate when nitrogen and phosphorus are not limiting.

The results presented herein consider nutrient availability as one explanation for the observed correlation between in situ A. hydrophila densities and trophic state. One objective of this aspect of the overall project was to determine the conditions required for the growth of seeded A. hydrophila in environmental water samples. The second was to examine the growth kinetics of A. hydrophila seeded into waters of varying quality. This was done in order to determine which, if any, of the growth parameters are correlated with the various physical, chemical or biological parameters (including the RTI) used in defining the trophic state of fresh waters.

MATERIALS AND METHODS

Growth Studies

Water samples for the growth studies were taken from various Rhode Island ponds at approximately 0.5 m depth and at selected limnetic sites away from the vicinity of aquatic marcrophytes. They were collected in acid-cleaned (overnight immersion in 10% HCl followed by six deionized-distilled water rinses), autoclaved, polypropylene containers. Upon return of the samples to the laboratory, those portions to be used in growth studies with raw (untreated) water were dispensed in 200 ml quantities to acid-cleaned, sterile, 500 ml Erlenmeyer flasks. Filtered-autoclaved water was obtained by passing portions of the samples through pre-washed (200 ml of deionized water), 0.22 μm GS Millipore filters (Millipore Corp., Bedford, MA.), at a vacuum of less than 13 cm Hg. The filtrates were dispensed to flasks as described; and the flasks were autoclaved at 121°C for 15 min, allowed to cool to room temperature, and vigorously shaken to aerate the water. They were then inoculated with the A. hydrophila test suspensions to a final density of approximately 10 organisms/ml, incubated at room temperature, and monitored periodically using the mA procedure for A. hydrophila (5). The interval between collection of the water sample and its treatment generally did not exceed six hours.

In those instances in which the ability of a given body of water to support the growth of the seeded A. hydrophila was examined against the "nutrient" levels in the water, 1 L water samples were collected from each of six, randomly selected, limnetic sites. These were pooled; the pool was filtered; the filtrate was dispensed into triplicate flasks; and the flasks of water were autoclaved and treated as described earlier.

The $\log_{10}$ densities of A. hydrophila were plotted against the incubation time in hours; two points during maximum exponential growth were identified; and these were used to calculate the generation (g) time using the formula

$$g = \frac{(t_2 - t_1) \ln 2}{\ln D_2 - \ln D_1}$$

where t_1 and t_2 and D_1 and D_2 are the incubation times in hours and densities per mil for the first and second points.

Inoculum Preparation and Maintenance

An A. hydrophila strain (N-1) isolated from a highly oligotrophic body of water (Bay of Naples, Maine) was selected for the growth studies. The cells from a nutrient broth culture of the strain (incubated for 18 h at 37°C) were washed three times by alternate centrifugation and resuspension of the cells in 0.85% NaCl. A flask of filtered, autoclaved, oligotrophic pond water was inoculated with an appropriate dilution of the A. hydrophila suspension and incubated as described above. Log-phase cells from this culture were serially passed into flasks of the same medium in order to provide a continuous supply of log-phase cells for use as the test inocula in all growth experiments.

Physical, Chemical and Biological Parameters

Six parameters were measured in assessing the trophic states of the ten Rhode Island ponds examined. They included hypolimnetic dissolved oxygen, water transparency (Secchi disc), total phosphorus, dissolved reactive phosphorus, inorganic nitrogen ($NH_3 + NO_2 + NO_3$ as N), and chloroyphyll a. Water temperature and conductivity were also measured. The six parameters were used in calculating a relative trophic index (RTI) for each body of water using a previously described procedure (1,2).

In situ dissolved oxygen, conductivity, and temperature measurements were made with a calibrated YSI model 54 oxygen meter (Yellow Springs Instrument Co., Yellow Springs, OH.) and YSI model 33 S-C-T conductivity and temperature meter, respectively. Water transparency was determined using a Secchi disc.

Samples for chemical analyses were collected at a "deep-station" with a messenger tripped, 1 liter, teflon-coated Van Dorn sampler (General Oceanics, Miami, FL.). Under conditions of stratification, two epi-, two meta-, and two hypolimnetic samples were taken; otherwise three samples were collected at representative depths. Samples were transferred into 500 ml acid-washed sample bottles. All samples were held at ambient temperature in the dark and processed in the laboratory within four hours.

Subsurface and pooled surface water samples for chemical and chlorophyll a analyses were treated as follows: A 100 ml portion of the sample was filtered through pre-washed (200 ml of deionized water) 0.45 µm HA Millipore filters. The filtrate was delivered into an acid-cleaned 125 ml polypropylene bottle and immediately frozen for future dissolved nutrient analyses; the filter was frozen for later chlorophyll a analysis. The second 100 ml portion was delivered (unfiltered) into an acid-cleaned 125 ml sample bottle and frozen for later total phosphorus analysis.

Colorimetric analyses for ammonia-nitrogen (NH_3 - N), nitrate-nitrite-nitrogen (NO_3 - NO_2 - N), dissolved reactive phosphorus (PO_4 - P), and total phosphorus (TP) were made using a Technicon Auto Analyzer II by the following methods (6); NH_3 - N, Berthelot reaction; NO_3 - NO_2 - N, copper-cadmium reduction followed by sulfanilamide diazotization, N-(1-napthyl)-ethylene diamine method; PO_4 - P, phospho-molybdenum blue method; TP, potassium persulfate digestion followed by PO_4 - P analysis. Fluorometric analysis of chlorophyll a utilizing 90% acetone extraction was according to Strickland and Parsons (7).

Statistical Analyses

The degree of association between variables was tested by the non-parametric Spearman rank correlation analysis utilizing the SAS computer statistical package (8).

RESULTS

Growth of the indigenous or seeded *A. hydrophila* at room temperature did not occur in the untreated (raw) water samples collected during the fall and early winter from eutrophic as well as oligotrophic waters in Rhode Island (Fig. 1). Pursuant to the identification and circumvention of those factors in raw water which prevented the growth of the seeded *A. hydrophila*, membrane-filtered (0.22 μm Millipore GS) water samples were examined for their abilities to support the growth of the seeded bacteria. This treatment was consistent with one objective of the study, the correlation of the growth kinetics of seeded *A. hydrophila* in the water to the concentrations of dissolved nutrients therein. However, membrane filtration alone was often found to be unsatisfactory for treatment of the water because the sterility of the filtrate could not be assured. When contamination of the filtrate occurred, the growth of *A. hydrophila* was generally retarded or precluded. Moreover, sterile filtrates, even from eutrophic bodies of water, did not always support growth of seeded *A. hydrophila*. This inhibitory effect could be eliminated by autoclaving the water at 121°C for 15 minutes. Therefore, the water samples used in all subsequent growth studies were filtered and then autoclaved. When the water samples were so treated, growth occurred; and relatively high densities of the organism resulted (Fig. 1).

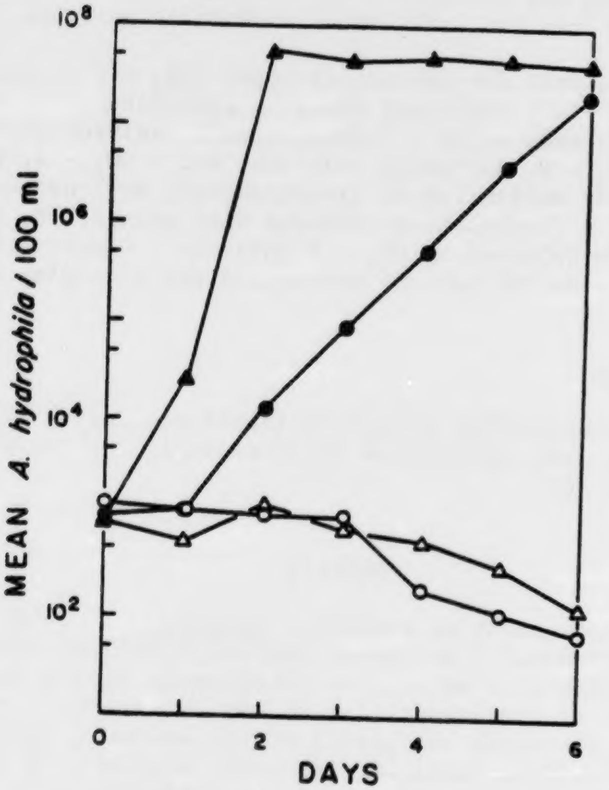

Fig. 1. Growth characteristics of A. hydrophila in untreated (o) and filtered-autoclaved (•) oligotrophic and in untreated (Δ) and filtered-autoclaved (▲) eutrophic pond waters.

Water samples from ten Rhode Island ponds were taken during the months of March through early June for the conduct of growth studies and the physical, chemical, and biological analyses necessary for trophic state assessment. The results from some typical growth experiments using filtered-autoclaved water samples from oligotrophic, mesotrophic, and eutrophic ponds are shown in Figure 2. It can be seen that lag periods were minimal or absent, presumably because cells from exponentially growing cultures in oligotrophic water were used as the inocula. Irrespective of the trophic state of the water used, the maximum densities (D_{MAX}) achieved in the growth experiments markedly exceeded those observed in situ during a previous summer; however, they were considerably less than those attained in nutrient broth (Table 1).

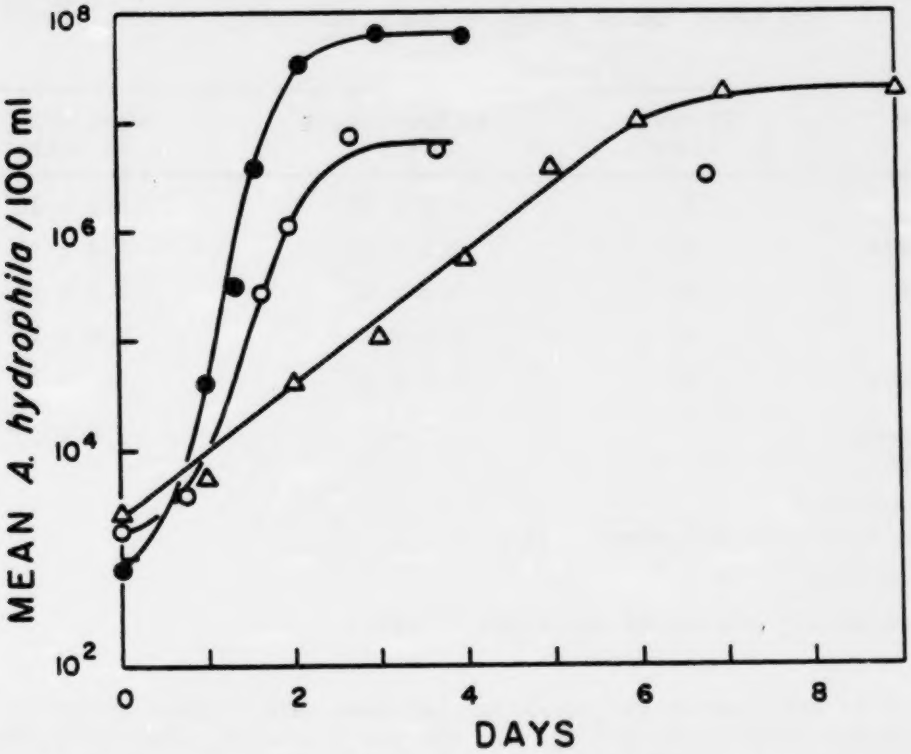

Fig. 2. Growth kinetics of A. hydrophila in filtered-autoclaved water from eutrophic (•), mesotrophic (o), and oligotrophic (Δ) water bodies, (points are means of 3 replicates). The mean generation times were 1.7 h, 3.5 h, and 10.3 h, respectively.

TABLE 1. COMPARISON OF IN VITRO, MAXIMUM GROWTH DENSITIES OF A. hydrophila FOR RHODE ISLAND PONDS TO THEIR RESPECTIVE IN SITU DENSITIES

Pond	Trophic State[a]	A. hydrophila in situ[b]	Density/100 ml in vitro[c]
Worden	E	2.0×10^3	1.4×10^7
30 Acre	E	1.5×10^3	1.3×10^7
Tucker	E	3.6×10^2	3.2×10^7
Deep	M	8.5×10^1	3.6×10^7
Yawgoog	M	8.5×10^1	2.1×10^7
Nutrient broth	-	-	3.8×10^{10}

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[a] E - eutrophic; M - mesotrophic

[b] From 1978 data (1)

[c] From growth studies as described in text.

Large differences in generation (g) time were evident among the ponds of varying water quality (Fig. 2), and the g time appeared to correlate with trophic state. Therefore, the degrees of association of g time and D_{MAX} achieved in the growth studies to those parameters indicative of trophic state (as well as the RTI) were examined using the Spearman rank correlation test. The correlation coefficients (r) and their significance (P) are shown in Table 2. Only the generation time was significantly correlated to any of the individual trophic state parameters or the RTI; the highest r value (0.91) obtained was for g time vs. the RTI calculated for the ten Rhode Island ponds using the National Eutrophication Survey's 209 lake data set (2). Highly significant correlations were also found for g time with total phosphorus and chlorophyll a; significant correlations were obtained with both Secchi depth and conductivity; no correlation was observed with dissolved inorganic nitrogen or dissolved reactive phosphorus.

TABLE 2. ASSOCIATION OF A. hydrophila GROWTH PARAMETERS TO PHYSICAL, CHEMICAL, AND BIOLOGICAL TROPHIC STATE MEASUREMENTS AND THE RTI

Parameter	r Value[a] for		Parameter	r Value[a] for	
	G[b]	Dmax[c]		G	Dmax
RTI (National base)[d]	0.91**	-0.18	$NH_3 + NO_2^- + NO_3^-$(as N)	-0.20	-0.17
RTI (Local base)[e]	0.82**	-0.23	Dissolved React. Phosphorus	-0.34	0.35
Total Phosphorus	-0.80**	0.34	Conductivity	0.77*	-0.06
Chlorophyll a	-0.81**	0.14	Dmax	-0.18	-
Secchi Disc	0.73*	-0.16			

* P <0.0005 ** P <0.0001

[a] Spearman rank correlation coefficients

[b] Generation time; [c] Maximum density achieved

[d] Relative Trophic Index based on ranking the 10 Rhode Island ponds using the 209 lake data base from the 1972 NES study (2)

[e] Relative Trophic Index based on ranking the 10 Rhode Island ponds among themselves

The longest generation time observed was 12.8 hours, and the shortest was 1.25 hours. The generation time for the nutrient broth culture, treated identically to the test cultures, was 0.56 h. Furthermore, from the generation time-RTI relationship shown in Figure 3, it would appear that a g time comparable to that observed with the nutrient broth cultures would not be achieved even in water samples from the most highly eutrophic waters.

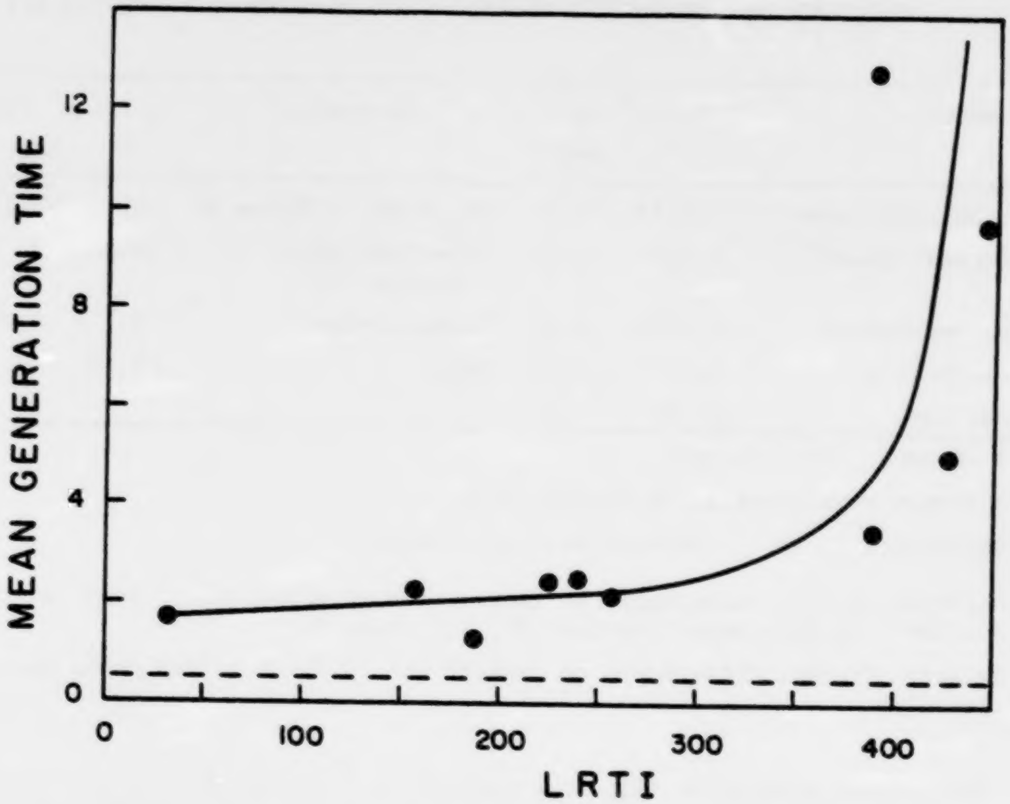

Fig. 3. Relationship of the generation (g) time for the growth of A. hydrophila to the Relative Trophic Index derived from the 10 Rhode Island ponds themselves (r=0.82, P<0.0005). Generation times for A. hydrophila are the means from three replicate growth experiments. The broken line is the g time in nutrient broth.

DISCUSSION

An earlier report described the relationship of the *in situ* *A. hydrophila* densities found within a given body of water to its trophic state (3). However, this bacterial measurement cannot be used in the late fall, winter, and early spring in temperate zones, presumably because of temperature limitations on the growth of the organism. A practical objective of the present study was to examine the growth characteristics of the organism in laboratory studies as an alternative or adjunct to the *in situ* bacterial measurements.

The mean generation time, as determined from growth studies using filtered, autoclaved water, appears to be a reasonable alternative to the *in situ* densities, especially for oligo- to early mesotrophic bodies of water. However, the discriminating ability of this measurement decreases as the waters become more eutrophic; and it would appear that, even in the most eutrophic water, the exponential growth rate would not be comparable to that obtained in nutrient broth. One possible explanation for this observation is that the availability of one or more specific nutrients (e.g. vitamins, amino acids, energy sources), other than those included in the RTI, limited the growth rate of A. hydrophila in these waters. The lack of correlation between the maximal density attained and any of those parameters indicative of trophic state may be explained in a similar way. This explanation is being pursued against the possibility that, once these nutrients are identified and added to the test waters, the generation times for eutrophic waters and the maximum densities achieved will be better correlated with trophic state and approach those densities observed in nutrient broth. Moreover, the supplemented water would then provide the "base medium" for examining the effects of added trophic state associated nutrients (e.g. nitrogen, phosphorus) on the growth of seeded A. hydrophila.

The lack of growth of A. hydrophila in any of the raw waters collected in Rhode Island during the fall and winter was a consistent phenomenon. It is possible that A. hydrophila is multiplying in these waters but that pressures such as predation, toxicity, competition, etc. are so intense that density increases are never observed. This explanation probably accounts for the observation that the maximum A. hydrophila density obtained in the growth studies for a given pond consistently exceeded the in situ densities observed during the summer. It is also consistent with the observation that the densities of seeded and indigenous A. hydrophila in raw waters are markedly reduced within 8-10 days, while the high densities achieved in filtered-autoclaved water from the same pond are maintained for extended periods of time, at least 23 days (data not shown). This aspect of the study also is being pursued. Some preliminary experiments are in agreement with the hypothesis that predation, toxicity, and competition are important in limiting the in situ A. hydrophila densities.

The generation time of A. hydrophila seeded into filtered-autoclaved water samples does show promise as a trophic state indicator for bodies of fresh water. This promise may be more fully realized for the generation time and even the maximum density attained in growth studies once more information is obtained on the nutrients which limit the growth of the organism in natural waters. Moreover, these growth parameters could be more indicative of trophic state than are the in situ densities of the organism since they are independent of predation, competition, toxicity, etc. In addition, as noted earlier, samples may be taken for assay during any season of the year.

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Comments on
Growth Kinetics of Aeromonas hydrophila in Fresh Waters and Their Relationship to Trophic State

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One of the major factors that causes deterioration of bodies of freshwaters is an increase in the trophic (i.e., nutritional) level. Such changes in the trophic state can result in enhanced growth of phyto- and zooplankton, in a decrease in the dissolved oxygen content with a concomitant decrease in the oxygen-requiring biota, in an increase in microorganisms including species pathogenic to animals, in production of toxic substance, in aesthetic deterioration, and in numerous other conditions that can render the waters unfit for consumption and recreational uses. Although increases in trophic states occur naturally, these are slow and long-term processes. More frequently, increases in trophic states are anthropogenic and usually result from inputs of sewage or industrial wastes. Because of the fragility of most freshwater systems, it is important that even subtle changes in trophic states be detected early and accurately.

Numerous methods, including chemical, physical, and biological assays, have been developed for detecting changes in trophic states. Although many of these methods, especially when used in combination, have been successful, most are time-consuming, expensive, and require specialized equipment. The development of a microbiological assay using a sensitive indicator species would eliminate many of these problems, as such assays are relatively rapid, inexpensive, reproducible, require relatively little equipment, and can be conducted by essentially untrained personnel.

The purpose of this research project is to develop and test such a microbiological assay, using the bacterial species, Aeromonas hydrophila. There are several characteristics that recommend the use of this species: it is a strict heterotroph and, therefore, dependent for survival on organic matter either introduced into or synthesized by autotrophs (which require inputs of inorganic nutrients) in the aquatic systems; it is a pathogen of human beings; and it does not form spores and, therefore, does not overwinter well nor survive long in the absence of nutrients. Consequently, the presence of A. hydrophila in waters can suggest that the nutrient loadings are of sewage origin.

Three other important factors that recommended this research project were that preliminary studies by the principal investigator had shown that densities of A. hydrophila were correlated with other indicators of enhanced trophic states (e.g., chlorophyll a and total phosphorus contents, turbidity, and the "relative trophic index"); the development, by the principal investigator, of a method for quantifying this bacterium in waters; and the competency, qualifications, and productivity of the principal investigator.

Inasmuch as this is a basic research project, the studies will extend beyond just demonstrating further the correlation between trophic states and densities of A. hydrophila in freshwaters. They will attempt to determine which specific nutrients in nutritionally-enriched waters are responsible for the enhanced growth of this specific indicator organism and whether there are significant nutritional differences between biotypes of this species which would differentially affect their survivial and growth. In addition, factors other than nutrition that could influence the density of the indicator organism (e.g., anti-microbial agents, predation) will be studied, inasmuch as such non-nutritional factors could indirectly affect population densities and, thereby, the correlation. The project will also determine whether the growth of A. hydrophila can be used to measure and predict the relative effectiveness of procedures to restore (i.e., decrease the trophic state) deteriorated bodies of freshwaters.

As with all research proposals, the review panel had a number of questions about this proposal. However, these were, for the most part, minor, and the concensus was that the principal investigator, given his experience and capabilities, would be able to resolve these.

In summary, the panel recommended support of this project, as it concluded that the proposal was scientifically sound, would result in significant new basic information, and that the Environmental Protection Agency would gain valuable information to aid it in assessing trophic states of freshwaters and which the Agency could use in its monitoring and regulatory functions.

DRILLING FLUID INDUCED CHANGES IN NEURONAL ACTIVITY MONITORED IN AN INTACT, BEHAVING MARINE ANIMAL

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INTRODUCTION

The greater need and utilization of domestic oil reserves has resulted in an increase in off-shore drilling particularly near the edge of the continental shelf. The importance of the continental shelf, and its associated coral reefs, to the ecological productivity of surrounding areas (1), has heightened concern over the environmental impact that such drilling operations pose to the surrounding fauna and flora.

The effects that oil-rig related pollution parameters have on the biota can be ascertained by subjecting the biota to various concentrations of one or more of the polluting parameters and monitoring changes in selected behavioral, physiological and developmental systems. This approach has been employed to determine the toxicity of such drilling-related environmental pollutants as petro-hydrocarbons (2, 3, 4, 5, 6, 7). More recently, research has begun to determine the toxic effects of drilling fluids on biota endemic to off-shore communities (8, 9, 10, 11, 12, 13, 14). Drilling fluids are heavy mixtures which can include fungicides, bacteriocides and pesticides and are used to facilitate drilling-related functions, e.g., drill-cutting removal and drill bit lubrication (15, 16).

We report here the first experiments to monitor changes in an animal's (the marine gastropod Mollusc, *Aplysia californica*) nervous system activity induced by drilling fluid. Our research utilized a recently developed extracellular electrophysiological recording technique (17) that makes it possible to monitor the neuro-physiological correlates of behavioral patterns in *intact, freely-behaving Aplysia* (18, 19). Changes in neuronal activity always precede behavioral changes and can occur without any visible behavior modifications (20). Therefore, detection of neuronal activity changes elicited by the presence of an environmental pollutant (such as drilling fluid) provides an important, sensitive method for determining toxicological effects below and above the threshold of observable behavioral modifications.

METHODS

Aplysia californica obtained from Pacific Bio-Marine Laboratories, Inc., were housed in 16 x 19 x 19 cm perforated plastic chambers within a 130-liter Instant Ocean tank (with automatic aeration and filtration) at 15-17°C under a 12:12 hr. light-dark schedule. Animals were fed dried sea weed and each was assigned a number (CC = chronic californica). Drilling fluid (Sample JX-FL 3-1 from July 11, 1980) was obtained from the Gulf Breeze Laboratories of the U. S. Environmental Protection Agency, Gulf Breeze, Florida. A 10 cc volume of 10 ppt whole drilling fluid (wet weight) minus tha barium sulfate was introduced via silastic cannulation tube (2 mm O.D) into the body cavity of experimental animals at 0.5 cc/sec. Control animals received either 5 or 10 cc of sterilized isotonic saline (sea water) by cannulation.

Surgery for implantation of the *in vivo* extracellular recording electrodes and cannulation tube was performed according to Kanz et al. (18). Following surgery, pre-operative body weight was restored by injecting sterilized isotonic sea water and the animal was allowed to recover overnight. The silastic cannulation tube was sutured to the internal body wall musculature. The monopolar electrodes were implanted on the *Aplysia's* siphon nerve, a peripheral nerve from the abdominal ganglion which innervates the siphon, an organ used in expelling sea water and excretory products from the mantle cavity, (Figure 1A). The double cuff electrode assembly (Figure 1B) consisted of two independent stainless steel wires each referenced to an indifferent lead in the chamber; each pair (i.e., monopolar and indifferent lead) was attached to the head stage of a differential amplifier. Signals from the amplifier passed through a 60 and 120 Hz Notch Filter (to eliminate some of the background noise), then to an oscilloscope, FM tape recorder and pen recorder. Thus, two independent electrophysiological records, were obtained from loci on the siphon nerve 10 mm apart (Figure 1C).

Two neurophysiological parameters were monitored: 1) the frequency and pattern of an identified burst of neuronal activity from the siphon nerve, Interneuron II (INT II) associated with a nearly simultaneous contraction of the gill, parapodia and siphon and believed to function in replacing "old" sea water with "new" in the mantle cavity (21, 22) and 2) the level of background spontaneous activity in the siphon nerve (Figure 1C).

The experimental protocol used 7 animals into which was cannulated 10 cc of 10 ppt. drilling fluid and 6 control animals 5 of which received 10 cc of sterilized saline and 1 received 5 cc of sterilized saline. The animals were monitored for 24 hours prior to drilling fluid (DF) or saline cannulation and a minimum of 24 hours after cannulation. Interneuron II

IN VIVO RECORDING

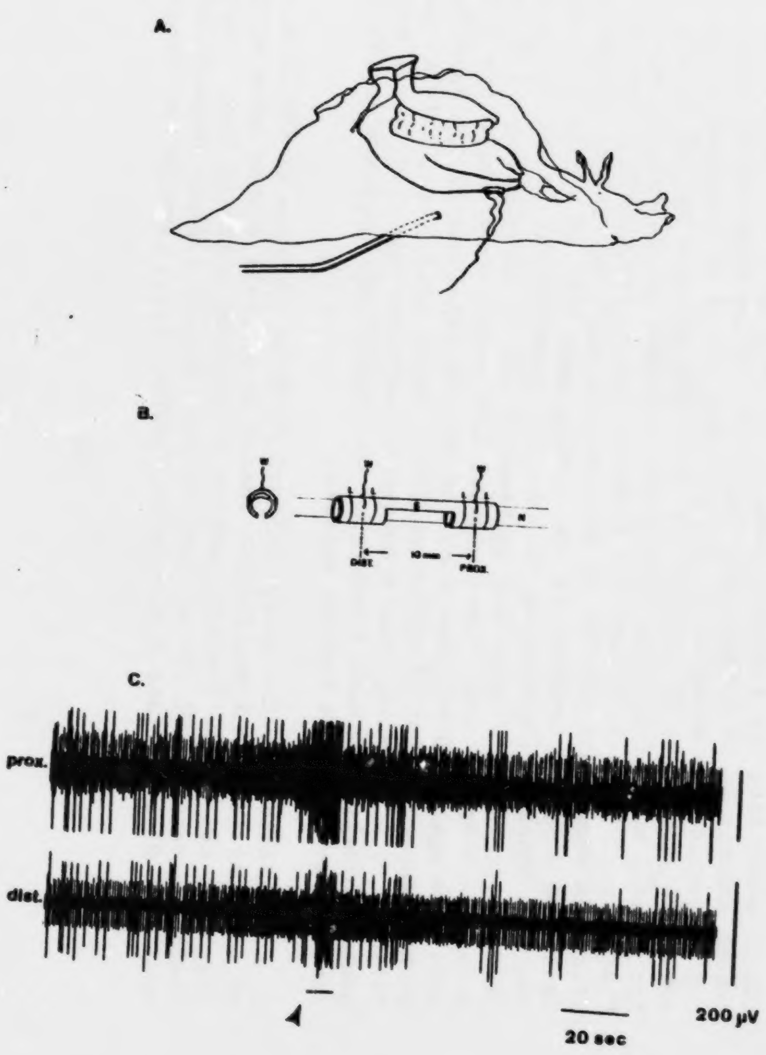

Figure 1. IN VIVO RECORDING

A. Schematic of Aplysia showing funnel-shaped siphon projecting dorsally between tissue (parapodia). Laminated gill is situated within the mantle cavity. The abdominal ganglion is sketched along with a double-cuff assembly in place on siphon nerve. Cannulation tube is seen emerging posteriorly (to left).

B. Schematic of double-cuff electrode assembly. Left-cross-sectional view of cuff where one of electrode wires (W) passes through silastic tubing. Opening at bottom represents trough in tubing to permit placement around siphon nerve. Right - Longitudinal view of double cuff assembly. N = nerve, L = ligature to afix assembly onto nerve, S = silastic tubing (O.D. = 1 mm). PROX. and DIST. refer to two independent monopolar electrode wires proximal and distal, respectively, to abdominal ganglion.

C. Five-minute example of analog double-cuff recording from siphon nerve of intact, unrestrained Aplysia. Both electrode records (prox. and dist.) displayed. Animal was quiescent but note the great amount of background spontaneous activity. Arrowhead indicates the occurrence of a spontaneous interneuron II (INT II) burst. An INT II burst consists of two phases: 1) increased activity from small and intermediate amplitude units and inhibition of large amplitude units (dashed horizontal line) and 2) decreased activity from small and intermediate amplitude units and rebound firing from large amplitude units (solid horizontal line).

burst frequencies were compared using the non-parametric Wilcoxon Matched Pairs, Mann-Whitney U and Spearman Rank correlation tests (23) at a confidence level of ≤ 5%, two-tailed.

RESULTS

Six of the 7 Aplysia administered 10 ppt drilling fluid (DF) died within 2 days whereas the 6 control animals survived a mean of 8 days following saline cannulation.

However, prior to death the experimental animals (i.e., DF cannulation) showed a significant increase ($N = 14$, $U = 8$, $p = 0.038$) in the number of Interneuron II (INT II) neuronal bursts per hour following DF cannulation ($\overline{X} = 11.97 \pm 13.29$) compared to the INT II burst frequency over the 24-hour period before DF cannulation ($\overline{X} = 4.94 \pm 11.17$). No significant change ($N = 12$, $U = 17$, $p = 0.93$) in INT II frequency was seen in the control animals before ($\overline{X} = 4.75$/hr ± 6.79) vs. after ($\overline{X} = 4.96$/hr ± 5.54) saline cannulation. The pre-cannulation and post-cannulation INT II burst frequencies (i.e., number per hour) for individual control and experimental animals is shown in Table I and Figure 2. Experimental animals showed a consistent and highly significant increase in INT II rate following DF cannulation. Control animals demonstrated inconsistent or insignificant changes in INT II rate after saline cannulation. Figure 2B also shows that the mean post-cannulation INT II rate seen in individual animals is correlated with the mean pre-cannulation rate ($N = 7$, $r_s = 0.92$, $p < 0.02$). Thus the animals with the highest, intermediate, or lowest ongoing INT II rate before introduction of DF also displayed the highest, intermediate, or lowest INT II rate, respectively, after administration of DF. An example of the hourly INT II burst rate for a control animal, CC 24 (Figure 3A), and an experimental animal, CC 19 (Figure 3B), before vs. after cannulation illustrates the large increase elicited by the presentation of DF compared to saline. Figure 3B shows that DF triggers an immediate increase in INT II bursts over the first 3 hours following cannulation compared to the ongoing mean INT II frequency of 9.0 ± 6.76 over the 24-hours before cannulation. In contrast, Figure 3A shows that the INT II rate immediately after saline cannulation decreased slightly compared to the ongoing mean rate of 5.32 ± 6.35 over the 24-hours prior to introducing saline. For experimental animal CC 19 (Figure 3B), the immediate DF-elicited increase in INT II frequency was followed by a decline over the next 6 hours (until 2200 hours) after which the INT II rate rose again and remained elevated for the remainder of the experiment. Such a time-course of DF effect on INT II burst rate (i.e., initial increase followed by a decrease, then a sustained increase) was seen in all experimental animals. No such substantial and significant increase in INT II rate was seen for the control animals.

In addition to the change induced by DF in the rate of INT II bursts, DF also resulted in a qualitative change in over-all siphon nerve activity. Figures 4 and 5 show 5-minute samples of neuronal activity from representative control (CC 18) and experimental (CC 19) animals respectively. Part A of Figures 4 and 5 give pre-injection (i.e., cannulation) neuronal

TABLE I. INTERNEURON II FREQUENCIES FOR CONTROL (SALINE) AND EXPERIMENTAL (10 PPT. DF) ANIMALS BEFORE VS. AFTER CANNULATION

Control Animals	$\overline{X}$ frequency (No./hr) before	after	N*	$Z^{\dagger}$	p^{ψ}
CC 7	2.0 ± 1.6	1.75 ± 0.89	51	1.10	<0.32
CC 13	4.46 ± 4.36	5.13 ± 5.93	52	0.83	<0.41
CC 18	8.34 ± 11.88	9.43 ± 8.64	78	2.30	<0.021
CC 24	5.32 ± 6.35	2.90 ± 4.15	77	2.39	<0.017
CC 26	2.64 ± 3.52	4.39 ± 3.89	77	2.53	<0.011
CC 27	4.48 ± 5.41	5.56 ± 5.98	77	1.22	<0.222
Experimental animals					
CC 11	1.96 ± 3.07	3.39 ± 3.51	78	2.28	<0.023
CC 12	3.18 ± 6.82	7.60 ± 10.4	78	3.71	<0.0002
CC 14	8.68 ± 11.04	20.28 ± 12.06	46	3.57	<0.0005
CC 19	9.0 ± 6.76	22.7 ± 16.21	78	4.78	<0.00006
CC 20	2.12 ± 3.02	5.55 ± 5.21	78	4.26	<0.00006
CC 21	3.54 ± 4.82	17.69 ± 12.85	53	4.97	<0.00006
CC 23	2.7 ± 2.61	13.93 ± 13.41	39	3.97	<0.0001

* N is the combined number of observations before and after cannulation

† Z is the statistical value computed from the Mann-Whitney test

ψ p is the calculated significance, two tailed; $p < .05$ is taken as significant.

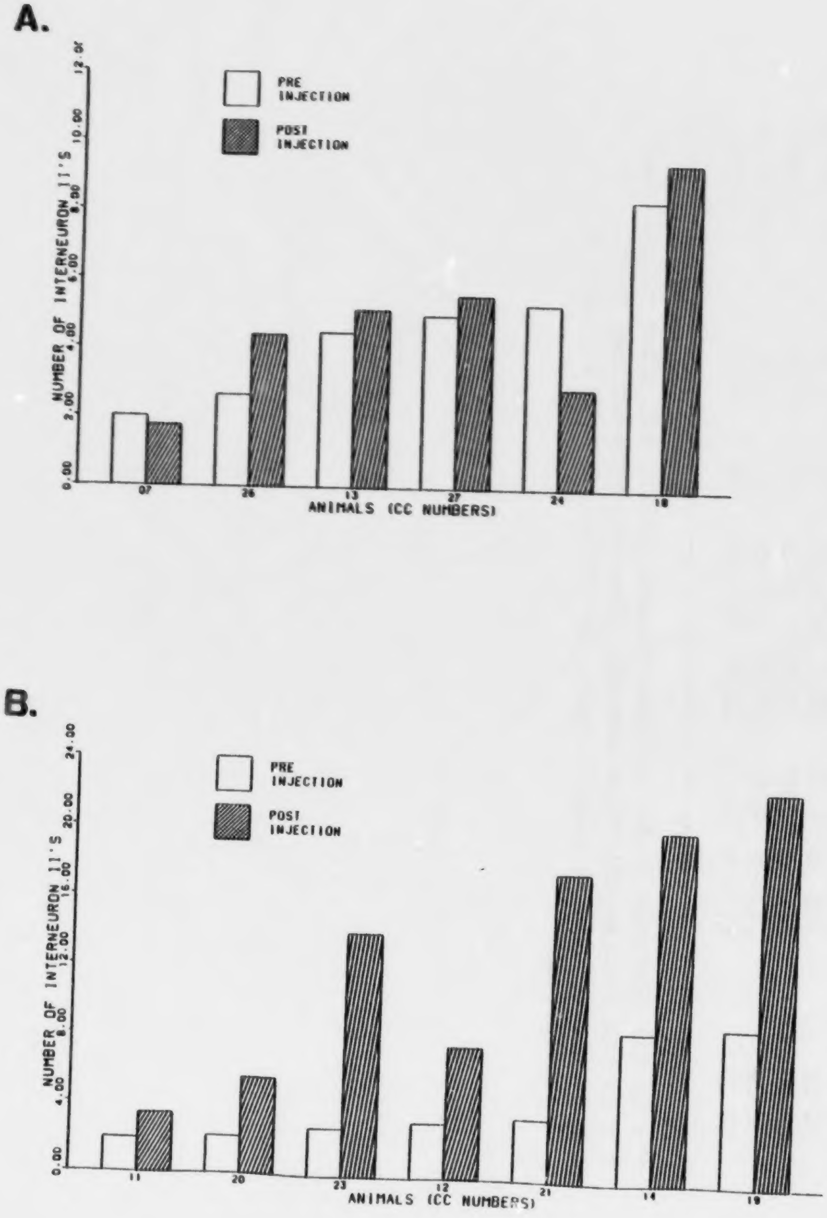

Figure 2.

A. Histogram of mean number of INT II bursts per hour before (unhatched) and after (hatched) cannulation of 5 cc or 10 cc of sterilized saline into six control animals (CC 7, CC 26, CC 13, CC 27, CC 24, CC 18). Animals were ranked along abscissa according to their mean pre-cannulation INT II burst rate. Post-cannulation INT II rate showed no tendency toward a correlation N = 6, $r_s = 0.34$, $p < 0.30$) with the respective pre-cannulation rate.

B. Histogram of mean INT II's/hr pre- and post-DF cannulation for seven experimental animals (CC 11, 20, 23, 12, 21, 14, 19,) ranked according to their pre-cannulation mean INT II rate. Pre-DF cannulation rate was positively correlated (N = 7, $r_s = 0.96$, $p <0.02$) with the respective post-DF cannulation rate.

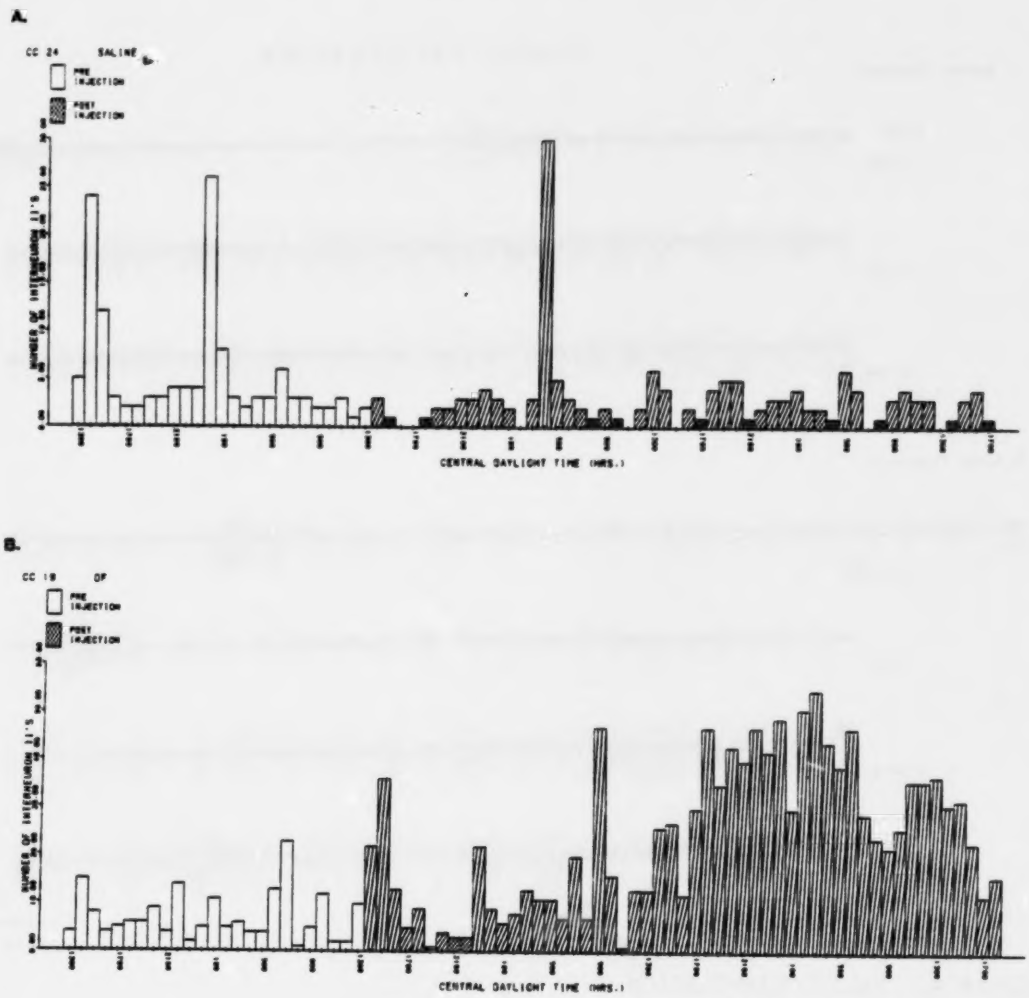

Figure 3.

A. Hourly histogram for a saline-control animal (CC 24) of number of INT II bursts over the 77-hour course of the experiment. Hatching (beginning at 1300 hours) represents the post-saline cannulation period. The INT II rate actually decreased for this animal following saline cannulation.

B. Hourly histogram for a DF experimental animal (CC 19) showing changes in the number of INT II bursts during the 77 hrs. of the experiment. Immediately after DF cannulation (i.e., within 40 sec.) the INT II rate increased significantly over the ongoing rate before cannulation; this increase lasted for 3 hrs. (until 1600 hrs.), declined and then returned to a significantly higher level (2200 hrs.) for the remainder of the experiment.

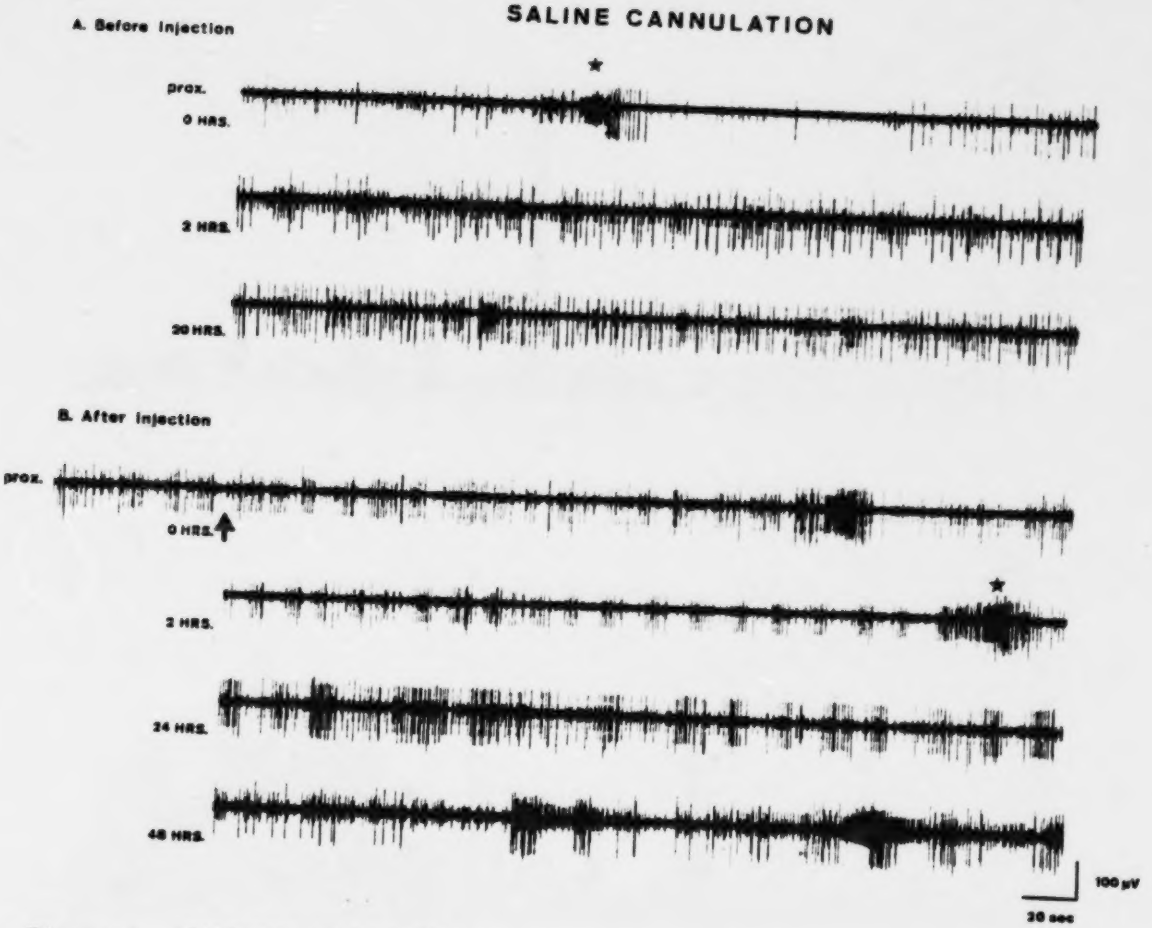

Figure 4. SALINE CANNULATION

A. Before Injection - Five-minute samples of siphon nerve activity (proximal cuff electrode only) from a control animal (CC 18). 0 HRS. (1300 hrs., CDT), 2 HRS. and 20 HRS. represent times after experiment began. Experiments were initiated one day after surgery for implantation of the double-cuff electrode assembly and cannulation tube. The star in the top record (0 HRS.) indicates an INT II burst. Characteristically, variability was seen in the general level of neuronal activity across the three samples.

B. After Injection - Four samples (proximal cuff record only) of siphon nerve activity over a 48-hr. period following cannulation (vertical arrow) of 10 cc of sterilized isotonic sea water into the body cavity of animal CC 18. Amplitudes of units were unchanged compared to samples in part A and the duration and pattern of INT II bursts (e.g., that "starred" in the 2 HRS. sample) showed no modification following administration of saline. The animal appeared healthy (based on the criteria of parapodia closed tightly and animal attached by foot to substrate) throughout the course of the experiment.

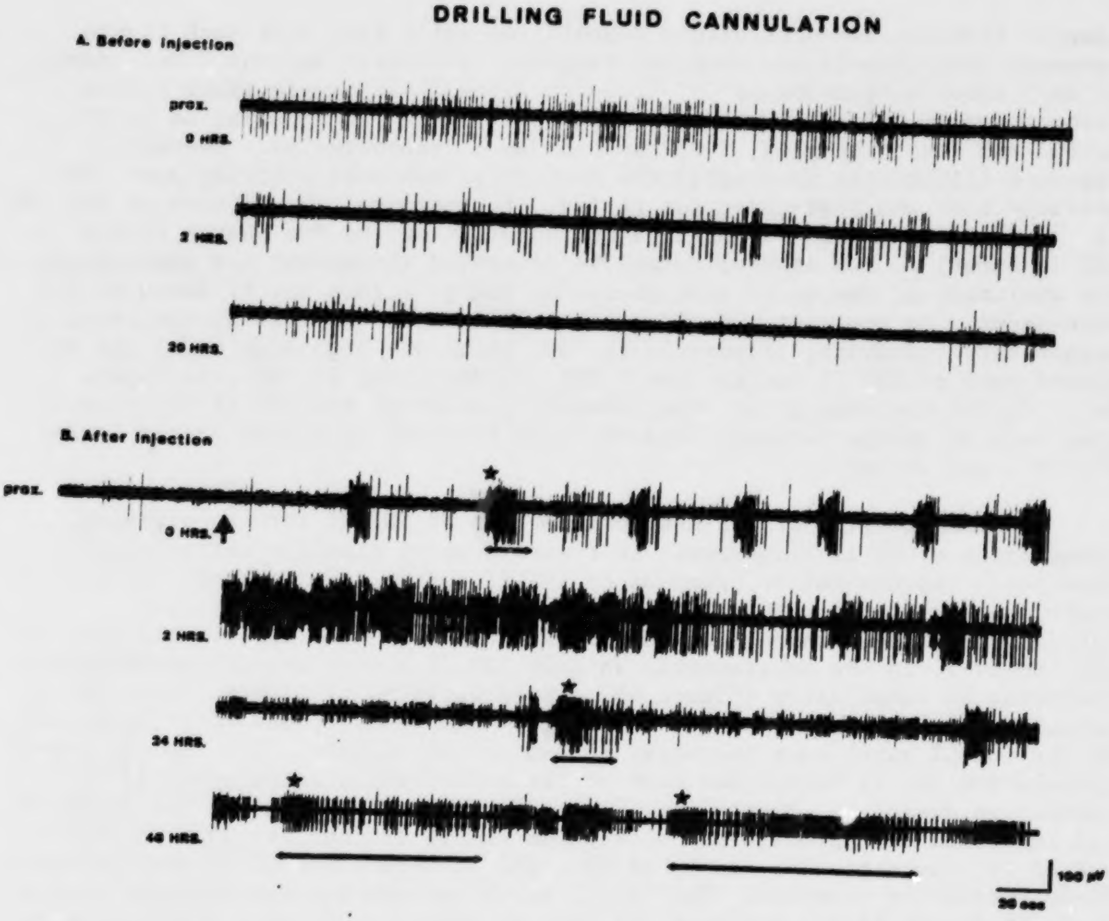

Figure 5. DRILLING FLUID CANNULATION

A. Before Injection - Three 5-min. samples of spontaneous siphon nerve (proximal electrode record only) activity from animal CC 19 prior to cannulation of drilling fluid. 0 HRS. corresponds to 1300 hrs., CDT. Variability was seen in the level of background spontaneous activity. No INT II bursts occurred during these sampling periods. Animal appeared healthy throughout this period.

B. After Injection - Four 5-min. post-DF cannulation samples of neuronal activity from animal CC 19. Following DF (10 ppt. wet weight) cannulation (vertical arrow in top sample) at a latency of approximately 40 sec., 7 INT II bursts were triggered within 5-mins. The duration (approximately 15 sec.) of the second triggered INT burst (star over first phase of burst in all samples) is indicated by a double-ended arrow. The level of background activity was also heightened. The second 5-min. sample (2 HRS., after DF cannulation) showed a continued high frequency of INT II bursts and heightened background activity. One day later (24 HRS.), background activity had decreased although the INT II burst frequency was still high; the duration of INT II bursts had now doubled to approximately 30 sec. ("starred" INT II burst with double-ended arrow). Two days (48 HRS.) following DF cannulation, unit amplitudes had decreased slightly and INT II burst durations had increased to 80-100 sec. Prolongation of both phases of the INT II burst characterized its extended time-course. By 48-hrs. post-DF cannulation, the animal's parapodia were not closed tightly; substrate attachment was still good.

samples from the 24-hours before cannulation while Part B of each figure presents post-cannulation neuronal samples. In Figure 4A, the first sample (0 HRS) shows a spontaneous INT II burst. The first sample after saline cannulation (0 HRS in Figure 4B) contains a single INT II burst as do the second and fourth samples (2 HRS and 48 HRS., respectively). Overall, Figure 4 illustrates no qualitative changes in neuronal activity over the pre-injection and post-injection period. Furthermore, the pattern of the INT II bursts (e.g., overall duration and duration of the two phases of the INT II burst) in the control animal is unchanged throughout the experiment; the amplitude of the units also showed no change across the 77 hours of the experiment. In contrast, DF elicited a qualitative increase in the level of siphon nerve activity, (Figure 5A vs. 5B) which was high-lighted by the enhanced rate of INT II bursts (see 0 HRS., 2 HRS., and 24 HRS., in Figure 5B). In the presence of DF, the neuronal pattern of the INT II burst was also seen to change (compare "starred" INT II burst in Figure 5B for 0 HRS., 24 HRS., and 48 HRS.).

The modification of the neuronal pattern of INT II bursts following cannulation of DF is illustrated in Figure 6 which displays (at a faster time-base) representative examples of INT II bursts for 3 control and 3 experimental animals. The INT II bursts for the control animals are basically unchanged throughout the course of the 77-hour experiments (Figure 6A); however, in the experimental animals INT II bursts changed substantially following DF cannulation (Figure 6B). Specifically, by 24 HRS., post-DF cannulation, the duration of both the first and second phase (cf. Figure 1C) of the INT II burst have increased in two of the animals. The typical pre-DF cannulation INT II bursts for each of the experimental animals was 10-15 seconds in duration. Twenty-four hours after administering DF, the duration had increased to approximately 35 seconds and 20 seconds for animals CC 14 and CC 19, respectively; and by 48 HRS. INT II bursts for CC 19 were typically 50-60 seconds in duration. The INT II burst pattern for experimental animal CC 21 in Figure 6B did not show the typical duration increase illustrated by animals CC 14 and CC 19; but the burst pattern did change in that by 24 HRS., post-DF cannulation, the large amplitude rebound units were not seen.

Therefore, the experiments demonstrate that 10 ppt DF: 1) is lethal (usually within 48 hours) to adult Aplysia californica, 2) effects a change in siphon nerve activity most notably a significant increase in the rate of INT II bursts, and 3) results in a change in the neuronal pattern of the INT II burst that precedes the first observable behavioral changes.

DISCUSSION

The Interneuron II burst seen in the siphon nerve is composed of activity from a population of motoneurons in the abdominal ganglion with processing in the siphon nerve that innervate the muscles of the siphon. (24, 18). Interneuron II has been shown to be a network of cells in the abdominal ganglion consisting of one or more burst-generator cells which are presynaptic on premotor cells which are in turn presumably presynaptic on siphon and gill motoneurons (22). The behavioral response (i.e., gill, parapodial and siphon

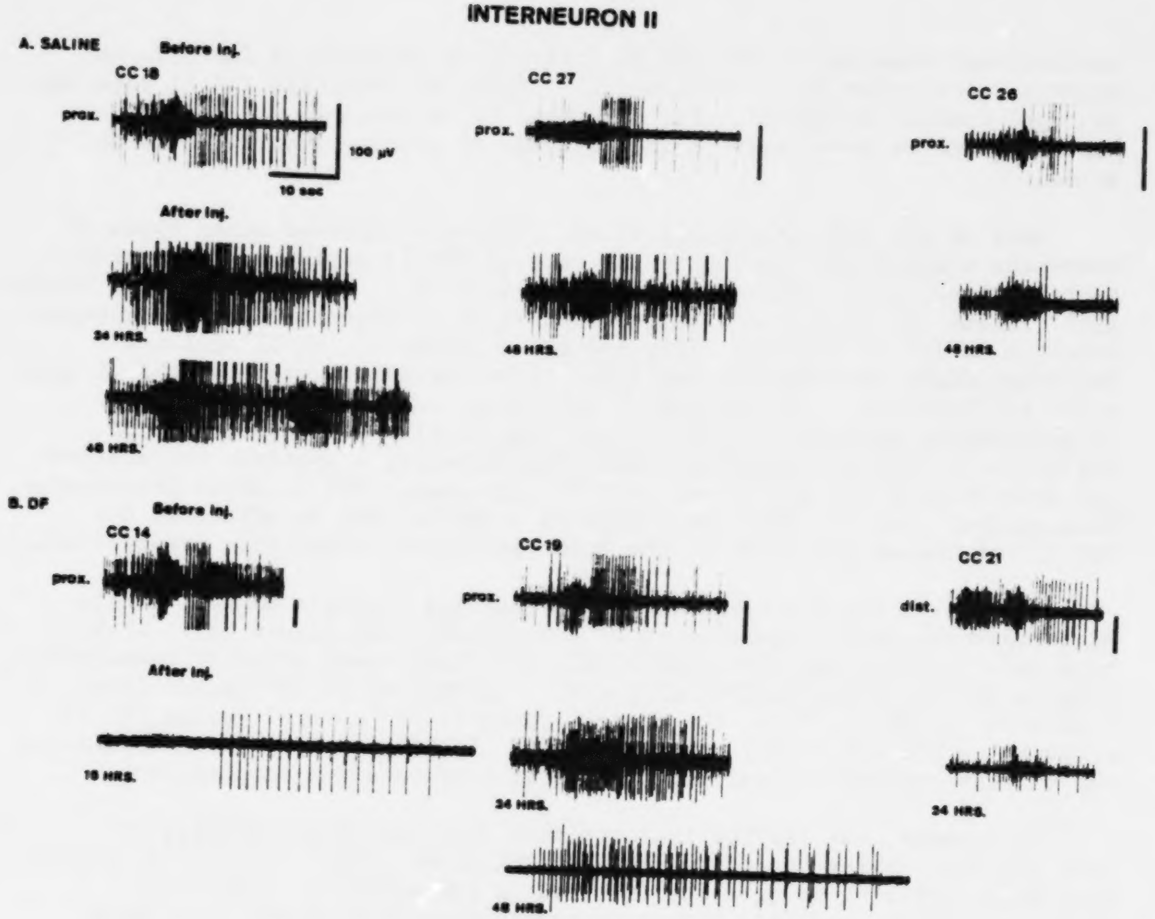

Figure 6. INTERNEURON II

Comparative illustration of time-course of INT II burst at faster time-sweep.

A. SALINE - INT II burst patterns and durations for control animals CC 18, CC 27 and CC 26 Before Inj. (injection) vs. After Inj. (injection) were within the range of variability typically seen in INT II bursts over time. Total burst durations were approximately 15-25 sec. consisting of phase one (approximately 2-5 sec.) and phase two (approximately 10-15 sec.).

B. DF - Three examples (from experimental animals CC 14, CC 19, CC 21) of INT II bursts were compared before (Before Inj.) and After Inj.) DF cannulation. Throughout the post-cannulation monitoring period INT II bursts became distorted. For CC 14 (16 HRS.) and CC 19 (24 HRS. and 48 HRS.), the INT II burst pattern was prolonged and each phase showed a considerably lower level of activity than before DF was presented. The change in INT II bursts in CC 21 was characterized by a slightly decreased overall duration and absence of rebound firing of large amplitude units in phase two.

contraction) associated with INT II activity is believed to function in Aplysia respiration and excretion (21). Thus, by recording INT II rate and neuronal changes in INT II burst patterns, it is possible to monitor the status of one or more physiological systems of primary importance to an animal.

Kanz et al. (18) and Eberly et al. (19) have reported other types of exogenous stimuli that influence the rate of INT II activity. For example, tactile siphon stimulation triggers short-latency (< 5 seconds) INT II bursts (18). Light and food presentation resulted in a longer-term (10-20 minutes) increase in INT II activity (19); and the increase in INT II activity following light presentation was found to be correlated with the INT II rate prior to lights-on. The results of this study show that DF induces a longer-lasting increase in INT II rate than tactile siphon stimulation, lights-on or food presentation. And significantly, a positive correlation was seen between the pre-DF and post-DF cannulation INT II burst frequencies. This suggest that DF, like the lights-on stimulus, may be effecting the INT II network at the level of the burst-generator rather than premotor cells.

Typically, the greatest and longest-lasting increase in INT II rate began approximately 5 hours after DF cannulation (see Figure 3B). A short-term rate increase was also seen within the first hours after DF cannulation (Figure 3B) but characteristically with a latency of 30-40 seconds (see Figure 5B, 0 HRS.). Thus, the animals appeared to "detect" (using INT II activity as the criterion) the presence of the DF soon after its cannulation but did not respond systemically for several hours after cannulation.

In summary, our results indicate that drilling fluid cannulation into the body cavity of Aplysia has a significant effect on neuronal activity that is believed to be directly involved in the animal's respiratory/excretory system; and this effect is manifested hours before observable deterioration in the animals condition.

We are currently bringing on-line computer procedures (25) for quantifying the firing pattern of individual units making up the analog spike train (e.g., as that seen in Figures 1C, 4, 5 and 6). This will make it possible to determine even subtler neuronal changes that might result from environmental toxins.

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Comments on
Drilling Fluid Induced Changes in Neuronal Activity Monitored in an Intact, Behaving Marine Animal

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ERL-Duluth

The above grant was recommended for funding primarily because using an electrophysiological technique to monitor neuronal response to stresses in an intact animal may provide a sensitive technique to assess toxicity to *Aplysia* and other organisms from drilling fluids and other pollutants. The respiratory pumping frequency, kidney efflux and locomotion behavior of *Aplysia* as affected by sublethal concentration of drilling fluid is to be determined. In addition, the influence of this potential toxicant on the activity of the siphon and pericardial nerves, the output from the network of cells in the abdominal ganglion responsible for respiratory pumping behavior, and the activity of abdominal ganglion cells capable of endogeneous bursting on neuronal activity will be studied.

Aplysia inhabit areas likely to be impacted by drilling fluids and other pollutants, have an accessible model nervous system from which neurobiological principles can be studied and extrapolated to other organisms and may be useful for predicting adverse effects to marine benthic organisms. The proposed work, although thought to have a high risk, was also thought to have a high gain potential. The ability to monitor neuronal output *in situ* from intact animals might prove to be a sensitive tool to detect sublethal effects.

Some of the criticism of the proposed work included the high concentrations of drilling fluids selected for the initial experiments and no intent to measure effects from individual components (i.e., barium sulfate, bentonite clay, chromium lignosulfonate, etc.) in the drilling fluids. The reviewers were also concerned about how neuronal-behavioral responses in the laboratory could be related to adverse effects in the marine environment.

QUALITATIVE ANALYSIS IN THE SETTING OF EAST COAST MARINE BENTHOS

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ABSTRACT

Soft sediment benthic ecology is described and forms the basis of six general, core qualitative models. Loop analysis (1) of these models indicates they provide robust predictions that are insensitive to the details of the models. The effect of predators and the use of specialized predators on three functional groups of benthic organisms do produce differences in predictions among the models. Seasonal variation is important to the benthos. The special considerations necessary for benthic environmental assessment are explained by the loop model analysis.

INTRODUCTION

We are examining benthic ecosystems along the western Atlantic from St. Margaret's Bay, Nova Scotia to Chesapeake Bay. Despite latitudinal ranges which produce differences in salinity, temperature ranges, tidal variation, sediment composition, etc., there is, on the whole, a similar benthic community. Although species composition does change along this gradient, with the region north/south of Cape Cod, Massachusetts frequently cited as the divide point, this difference is marginally important when considering species aggregated into functional groups. Consequently, we are presenting a caricature of the benthos that will illustrate the special considerations for the western Atlantic. At any specific location, a detailed description of the benthic community is necessary.

Our theoretical models highlight the benthic organism interactions likely to give counter-intuitive results. It alerts us to look for the surprises in environmental effects. The general models are not intended to surplant local information and detailed measurements. Rather, it tells us the kinds of local information that can aid environmental assessment, which measurements must be made, and which can be ignored because they don't change the predicted outcome.

Our general benthic models do include particular data. First, the models are restricted to the soft-bottom sediments: sand, sand-mud and mud. Second, the models consider patches within an estuarine bay, tidal flat, or a small part of a community along the shallow continental shelf. Thus, Buzzard's Bay in southeastern Massachusetts is about 19.5 kilometers wide and 46 kilometers long. Within this area are patches of "identifiable" dominant organisms. For example, Sanders (2, 3) identifies especially dominant burrowing polycheate-small bivalve areas as Nephtys-Nucula communities. Mills (4) finds areas in nearby Barnstable Harbor dominated by Ampelisca, a tube-building amphipod. Any benthic ecosystem will contain a variety of patches with an individual patch expanding, shrinking or becoming extinct (5). The consequence of scale, i.e., models for patches versus entire bays, will be considered below especially in the effect on community or subcommunity stability.

BENTHIC MODELS

Soft-bottom benthic organisms can be divided into five functional groups (6) according to the way in which organisms alter the environment. Through sediment mediation, benthic animals may negatively effect one another. Oversimplified, there are organisms which bind together particles, hence, stabilize the soft sediment substrate versus those which disrupt or resuspend material into the water column, hence, disturbing sediments. Organisms which bind sediments are primarily tube-building annelids and crustaceans. Many vascular plants or submerged aquatic vegetation with complex root systems can be classified as sediment stabilizers. Destabilizers include all mobile burrowing animals like bivalves, gastropods and many polychaete worms which move either just under the sediment surface or forage deep in the sediments.

Model 1 (Figure 1) represents this simple view of benthic ecology. Double negative links between the two functional groups indicate a competitive relationship. Tube-builders construct vast arrays of tubes, resembling a mat. Mats inhibit or prevent foraging of the mobile-burrowers (3, 7). Constant sediment reworking by mobile burrowers inhibits the ability of tube-builders to construct tubes or to get established in an area (3, 7, 8).

Densities of both tube-builders and mobile-burrowers effect their own population growth rate, hence, are self-damped (1). The pool of baywide larval production is independent of adult populations in the patch. But larval settlement is necessary for survival of either functional group in the patch. This input of larvae is the reason for self-damping. See (1) for mathematical explanation. Tube-builders are also self-damped because of migration or increased mortality as a consequence of increasing density.

The next four models increase in complexity over Model 1 by the addition of the functional group filter-feeders. Filter-feeders are composed of filter-feeding bivalves such as Mulinia laterialis, Gemma gemma or Mya arenaria. Filter-feeders interact only with tube-builders in Model 2. Models 3 through 5 differ from Model 2 by including alternative interactions of filter-feeders with mobile-burrowers. Assumptions about all the models are listed in Table 1.

TABLE 1. MODEL ASSUMPTION LIST

Model Assumption First Occurs	Assumption No.	Description of Assumption
2	1	Subsystem TB-MB is unstable when part of a larger community that has filter feeders present. Mills (4) showed that mobile burrowers, *Nassarius*, did not occur with tube builder, *Ampelisca*.
2	2	Subsystem FF-TB is stable. Mills (4), Watling (14) have shown that *Gemma gemma* increases when Ampelisca is present, but not with mobile burrowers.
3	3	(-) effect of DF on FF times self damping on TB is greater than (+) effect of MB on FF through TB. In the mud environment, this corresponds to the trophic amensalism hypothesis (8).
3	4	(-) effect of MB on TB times self damping on FF is greater than (+) effect of MB on TB through FF. In mud areas direct negative effects of disturbance on TB by MB times strong self damping on FF would be stronger than the strong effect of MB on FF times the relatively weak effect of FF on TB due to larval filtration. TB larva not very succeptible to filtration in mud sediment with a low density of suspension feeders.
4	5	Assumption 1 plus the assumptions that (-) effect of FF on both TB and MB are equal. In sand, filter feeders can occur in dense assemblages and probably have an equal effect on TB and MB through filtering of larvae.
4	6	Assumptions 1 and 2 combined: the (-) effect of TB on MB times self damping on FF is greater than (-) effect of TB on FF times (-) effect of FF on MB. Extended, this assumption also means that because the (-) effect of FF on MB and FF on TB are equal (assumption 1), and the effect of FF on TB is less than the self dampong on TB (assumption 2) and because the self damping on TB is less than the effect of TB on MB; the

TABLE 1. ....CONTINUED

Model Assumption First Occurs	Assumption No.	Description of Assumption
		effect of TB on MB is greater than the effect of FF on MB. Also, the effect of TB on MB is composed of adult-adult and adult-larval interactions, whereas the effect of FF on MB is composed solely of an adult-larval interaction.
5	7	Note that the (-) effect of DF on FF is one way due to trophic amensalism hypothesis; (-) effect of FF on MB is adult-larval filtration.
6	8	Assumes the positive feedback subsystem of MB and "Free mud" is a stable system.
6	9	Needs to be verified; results under this assumption could not be clearly seen with information at hand. Interactions need to be relatively quantified.
9	10	The (+) effect of "Free sand" on TB times the self damping on FF is greater than the (=) effect of "Free sand" on FF times the (-) effect of FF on TB. If we assume sand helps TB and FF equally, we can see that in a mud patch, FF will not have a strong siphoning effect on TB, at least not as strong as rate of input of FF to patch (i.e., self damping on FF).
5	11	The (-) effect of TB on MB times the (-) effect of MB on FF (i.e., the positive effect of TB on FF through MB) is greater than the direct (-) effect of TB on FF times the self damping effects of MB on itself. Link TB on MB is assumed strong relative to self damping on MB, and the trophic amensalism effect of MB on FF is probably stronger than the adult-larval-mat interaction between TB and FF.

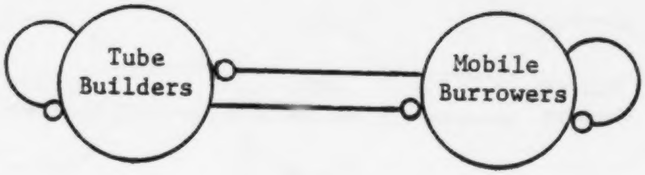

Tube
Builders
Mobile
Burrowers

Figure 1. Model 1, two benthic functional groups.

TABLE 2. MODEL 1 PREDICTIONS ON DIRECTION OF CHANGE IN ABUNDANCE THROUGH CHANGE IN RATE OF GROWTH

(+) Input to	Effect on Tube Builders	Effect on Mobile Burrowers
Tube Builders	+	-
Mobile Burrowers	-	+

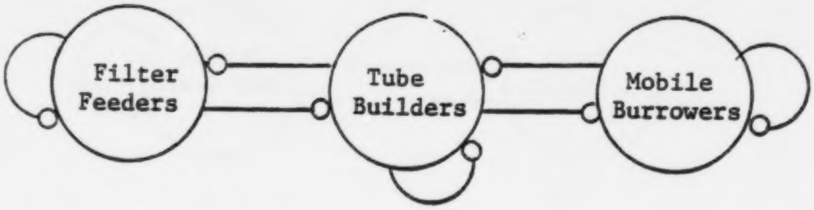


Figure 2. Model 2, an expanded version of model 1. Filter-feeder functional group in competition with tube-builders.

TABLE 3. MODEL 2 PREDICTION ON DIRECTION OF CHANGE IN ABUNDANCE THROUGH CHANGE IN GROWTH RATE. SIGNS IN PARENTHESES INDICATE ASSUMPTIONS WERE MADE ABOUT RELATIVE MAGNITUDE OF LINKS. ASSUMPTIONS ARE SUMMARIZED BY SUBSCRIPT IN TABLE 1.

	Effect on		
(+) Input to	Filter Feeders	Tube Builders	Mobile Burrowers
Filter Feeders	$(-)_1$	-	+
Tube Builders	-	+	-
Mobile Burrowers	+	-	$(+)_2$

Model 2 (Figure 2) has interference between tube-builders and mobile-burrowers as in Model 1. In addition to the reasons for interference stated previously, Woodin (9) adds that settling larvae are consummed by tube-builders or are buried in tube-builders fecal material. Thus, tube-builders negatively effect mobile-burrowers and filter-feeders. On the other hand, settling tube-builder larvae are unable to construct tubes, often become buried in the very flocculent upper sediment layer, and are possibly consumed by foraging burrowers.

In dense assemblages, filter-feeders can effectively siphon the majority of larva (even their own) attempting to settle in their area (9). Therefore, filter-feeders negatively effect tube-builders. Because of a high rate of larval recruitment from outside the patch, filter-feeders are self-damped; density-dependent consumption of their own larvae enhances this self-damping.

Sediment composition can be, roughly speaking, either mud, sand, or sand-mud and the benthic communities of each are represented in Models 3, 4 and 5, respectively.

In muddy areas, mobile-burrowers rework the sediments, resuspending the upper layers of the sediment into the water column. Floculent material interferes with the respiratory and feeding mechanisms of filter-feeders (8). Thus, mobile-burrowers will negatively effect filter-feeders, and this is depicted in Model 3 (Figure 3).

A sandy area, as represented in Model 4 (Figure 4), reverses the negative link of Model 3; now, it is from filter-feeders to mobile-burrowers. Sand is not easily resuspended into the water column, but it is on excellent substrate for and is often found with dense assemblages of filter-feeders. In high abundance, filter-feeders may increase mortality on mobile-burrowers' settling larvae (9).

At a mixture of sand-mud, the filter feeders and mobile-burrowers are true competitors negatively effecting each other. Thus, Model 5 (Figure 5) has a negative pair of links between all the functional groups.

Model 6 (Figure 6) distinguishes direct adult-larvae interactions from sediment mediated interactions. Restricted to a mud sediment, as in Model 3, we include two sediment variables. Free-mud and free-sand represent the percentage of mud and sand not amalgamated into a tube or tube mat. Free-mud benefits mobile-burrowers, facilitating foraging. The mobile-burrowers disrupt tubes hence "freeing" mud; mobile-burrowers and free-mud have paired mutualistic links. The negative link from mobile-burrowers to tube-builders is larval mortality, unlike Model 3 and 5 where it includes adult-adult interactions via sediment disturbance.

Tube-builders cannot construct durable tubes in mud alone. Tube-mat density is highest in sand-mud environments, increasing sharply in mostly mud habitats (2). Therefore, free-mud inhibits tube-builders, while free-sand assists them. Tube construction, of course, decreases free-mud and free-sand. Tube mats, although no different in particle composition than the original substrate, nevertheless alter sediment physical characteristics, changing

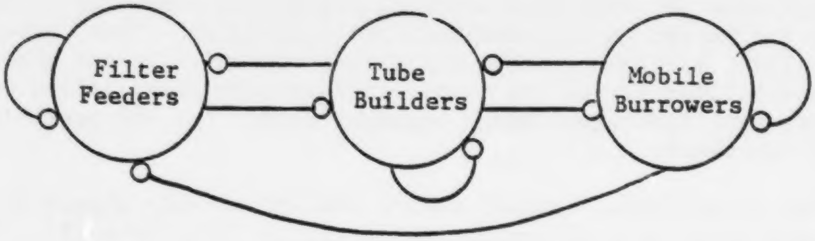

Filter Feeders
Tube Builders
Mobile Burrowers

Figure 3. Model 3, the same as model 2 but includes negative interaction of mobile-burrowers on filter feeders.

TABLE 4. MODEL 3 PREDICTIONS ON DIRECTION OF CHANGE IN ABUNDANCE THROUGH CHANGE IN RATE OF GROWTH. SEE TABLE 3 CAPTION FOR EXPLANATION OF PARENTHESIS SIGNS AND SUBSCRIPTS

	Effect on		
(+) Input to	Filter Feeders	Tube Builders	Mobile Burrowers
Filter Feeders	$(-)_1$	-	+
Tube Builders	?(+)	+	-
Mobile Burrowers	$?(-)_1$	$?(-)_2$	$(+)_2$

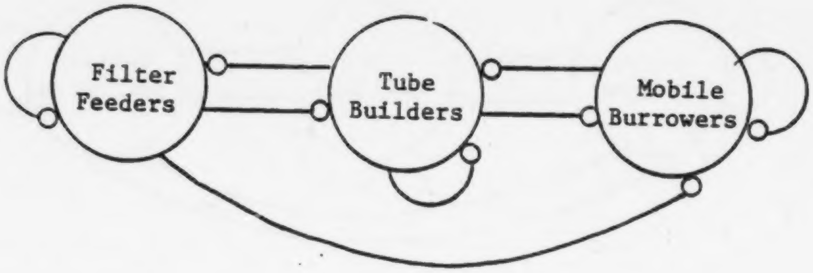

Filter Feeders
Tube Builders
Mobile Burrowers

Figure 4. Model 4, with reverse direction from model 3 of negative interaction between filter-feeders and mobile-burrowers.

TABLE 5. MODEL 4 PREDICTIONS ON DIRECTION OF CHANGE IN ABUNDANCE THROUGH CHANGE IN RATE OF GROWTH. SEE TABLE 3 CAPTION FOR EXPLANATION OF PARENTHESIS SIGNS AND SUBSCRIPTS

	Effect on		
(+) Input to	Filter Feeders	Tube Builders	Mobile Burrowers
Filter Feeders	$(-)_1$	$(+)_5$	$(+)_5$
Tube Builders	-	+	$(-)_6$
Mobile Burrowers	+	-	$(+)_2$

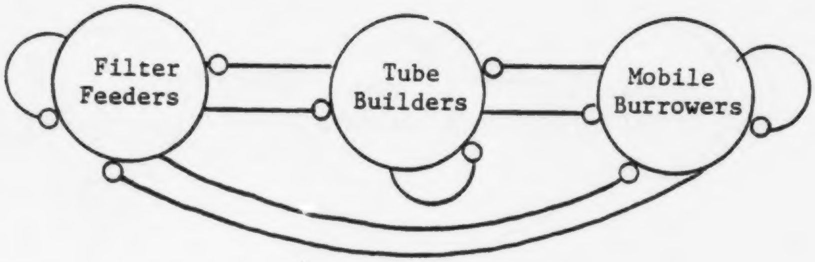

Filter Feeders
Tube Builders
Mobile Burrowers

Figure 5. Model 5 includes all pairwise competitive links between functional groups.

TABLE 6. MODEL 5 PREDICTIONS ON DIRECTION OF CHANGE IN ABUNDANCE THROUGH CHANGE IN RATE OF GROWTH. SEE TABLE 3 CAPTION FOR EXPLANATION OF PARENTHESIS SIGNS AND SUBSCRIPTS

	Effect on		
(+)Input to	Filter Feeders	Tube Builders	Mobile Burrowers
Filter Feeders	$(-)_1$	$(+)_5$	$(+)_5$
Tube Builders	$?(+)_{11}$	$(-)_7$	$(-)_6$
Mobile Burrowers	$(-)_1$	?	$(+)_2$

permeability and hydrodynamics (4, 10). Free-sand can increase filter-feeder populations either through inducing larval settlement or curtailing mud resuspension.

Filter-feeders and tube-builders have adult-larvel interactions (9) as in Model 3. There is, however, a long negative path of tube-builders through free-sand on filter feeders. Physiological/morphological constraints of sand-mud substrate on filter-feeders become excentuated by tube-builder activity. Thus, it may be that benthic organism activity creates patchiness in nearly homogeneous physical regimes.

As in Model 3, mobile-burrowers resuspend sediments that clog filter-feeder apparatus. Model 6 includes direct negative links from mobile-burrowers to filter-feeders for easy comparison with Model 3. The negative link should, however, be from free-mud to the filter-feeders. Predictions will not change if we redraw the negative link because the mobile-burrowers and free-mud variables are symmetrical in the model. Thus, this model cannot distinguish between which negative link or if both should be present. If it seems important to do this, then another model must be developed which lumps variables according to other criteria such as separating adults from larvae.

MODEL PREDICTIONS

Tube-builders bind sediments while mobile-burrowers disrupt sediments; these two functional groups are interference competitors. Competition can lead to potential instability through positive feedback. On the other hand, negative feedback of the effects on the two organismic types enhances the likelihood of co-occurrence. Broadly speaking, any soft-sediment patch will have tube-builders and mobile-burrowers (Sanders, pers. comm.). In the absence of filter-feeders, another competitive group, we assume the two former groups are part of a stable community; hence the overall community has negative feedback. In the presence of the filter-feeders, we reverse this assumption, as will be pointed out in the analysis of our later models. All assumptions made are listed in Table 1 and refer to subscripts in all subsequent tables.

For Model 1, we show that (Table 2) a positive change in the population growth of tube-builders will increase the tube-building fauna while decreasing the mobile-burrowing fauna. For short, a positive (or negative) change in the growth rate of a variable we call a "parameter input." Likewise, a positive parameter input to mobile-burrowers increases their own abundance and decreases that of the tube-builders. This is the stereotypic pure competitive interaction result.

Model 2 recognizes a third faunal benthic group, the filter-feeders. This group siphons larvae of both mobile-burrowers and tube-builders, reducing self-damping especially on mobile-burrowers. However, community stability requires strong self-damping. The mobile-burrower/tube-builder subsystem is probably unstable when imbedded in a larger community containing filter-feeders. Indirect support for this assumption comes from Mills' (4)

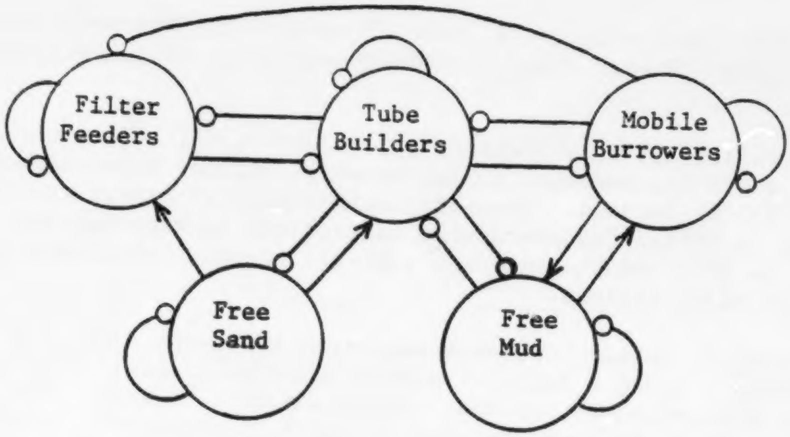

Filter Feeders
Tube Builders
Mobile Burrowers
Free Sand
Free Mud

Figure 6. Model 6 which includes physical characteristics of the sediment as the variables free-sand and free-mud.

TABLE 7. MODEL 6 PREDICTIONS ON DIRECTION OF CHANGE IN ABUNDANCE THROUGH CHANGE IN GROWTH RATE. SEE TABLE 3 CAPTION FOR EXPLANATION OF PARENTHESIS SIGNS AND SUBSCRIPTS

	Effect on				
(+) Input to	Filter Feeders	Tube Builders	Mobile Burrowers	Free Sand	Free Mud
Filter Feeders	$(-)_1$	$(-)_8$	+	$(+)_8$	+
Tube Builders	?	$(+)_8$	-	$(-)_8$	-
Mobile Burrowers	(+)	$(-)_9$	$(+)_2$	$(+)_9$	$(+)_9$
Free Sand	?	$(+)_{10}$	$(-)_{10}$	$(-)_{11}$	$(-)_{10}$
Free Mud	?	?	+	?	?

observation that tube-building amphipods of the genus Ampelisca do not coexist in Barnstable Harbor with the burrowing gastropod Nassarius.

Filter-feeders siphon their own larvae, tube-builders show adult density-dependent effects, so both probably have strong self-damping. Gemma gemma, the filter-feeding bivalve, is abundant when tube-building Ampelisca is active and mobile-burrowers are relatively rare (4). Consequently, we assume the filter-feeder/tube-builder subsystem is stable.

Because we assume an unstable tube-builder/mobile-burrower subsystem, a Model 2 prediction (Table 3) is non-intuitive. An environmental change that increases the growth rate of filter-feeders causes a decrease in their own abundance. Of course, their turn-over rate has increased. A question mark is placed in the table and a negative sign in parentheses to emphasize that an assumption is made about relative strengths of links. In a following section, we describe the kinds of data that are necessary to test the assumption.

The fine mud sediment region of the benthos is described in Model 3. Mobile-burrowers disturb the mud, clogging filter-feeder siphons (8, 9). Model 3 predictions (Table 4) agree closely with that for Model 2 (Table 3) except that three question marks appear. These question marks, unlike that of Model 1, are not stability assumptions but occur because at least two pathways exist from one species group to another with opposite effects. For example, a parameter input entering through mobile-burrowers can negatively effect filter-feeders through a direct path and positively via tube-builders.

As in the first three models, we have calculated predictions for Models 4, 5, and 6 (Tables 5, 6, 7).

All six model predictions are summarized in Table 8. The models all agree in the effect of a parameter input to tube-builders on mobile-burrower abundance and the effect of a parameter input on mobile-burrowers on their own abundance. There is nearly complete agreement, except for Model 5, in predicting abundance change to tube-builders through a parameter input to themselves. And, while Model 3 and 5 give ambiguous predictions, all other models agree in predicting a negative change in tube-builder abundance from a parameter input to mobile-burrowers. The fact that added model complexity conforms with a simple model's predictions is reassuring.

The five complex models predict a positive input to filter-feeders causing a decline in their abundance. This is a consequence of the single assumption that the tube-builder/mobile burrower subsystem, in the presence of filter-feeders, is unstable. Even if this is untrue, all the models predict the same result, only reversed from above. To get different results between these models requires that either (a) the parameter input to filter-feeders is so strong as to swamp network effects in some patches, but not others, or (b) there exists an unspecified unstable subcommunity which links only with filter-feeders and that this is true in some patches and not others, or (c) in some patches, the tube builder/mobile burrower subcommunity is stable, and in other patches it is unstable. This last possibility is the most likely, and underscores the caution that these general models cannot be applied indiscriminately for environmental assessment, but must be verified according to local conditions.

TABLE 8. SUMMARY OF ALL SIX CORE BENTHIC MODEL PREDICTIONS. A SIGN IN PARENTHESIS FOLLOWED BY AN * INDICATES THE MODEL IS FORCED TO THIS PREDICTION. A (?)* MEANS FORCING CHANGES THE INDICATED PREDICTION TO AMBIGUOUS PREDICTION

	Model No.	Filter Feeders	Tube Builders	Mobile Burrowers
Filter Feeders	1	n/a	n/a	n/a
	2	-	-	+
	3	-	-	+
	4	-	+	+
	5	-	(+)*	+(?)*
	6	-	-	+
Tube Builders	1	n/a	+	-
	2	-	+	-
	3	+(?)*	+	-
	4	-	+	-
	5	+(?)*	- (?)*	-
	6	?	+	-
Mobile Burrowers	1		-	+
	2	+	-	+
	3	-(?)*	(-)*	+
	4	+	-	+
	5	-(?)*	(-)*	+
	6	+	-	+

Question marks in Model 3 and 5 can be used to make predictions about community interactions rather than assembling them in counter-point to the above. We call this "forcing the model" because we presume Models 1, 2, 4, and 6 give the "correct" result, "force" the question marks to give the same result, and examine the consequence of doing so. Hence, replacing the question marks in the mobile-burrower/tube-builder box with a negative sign implies the negative effect of mobile-burrowers directly on tube-builders outweighs the benefits to the tube-builder by having its filter-feeding competitors reduced. This conclusion is reasonable since we already have indicated competition between mobile-burrowers and tube-builders is strong and that filter-feeders have strong density-dependent effects.

BENTHIC PREDATORS

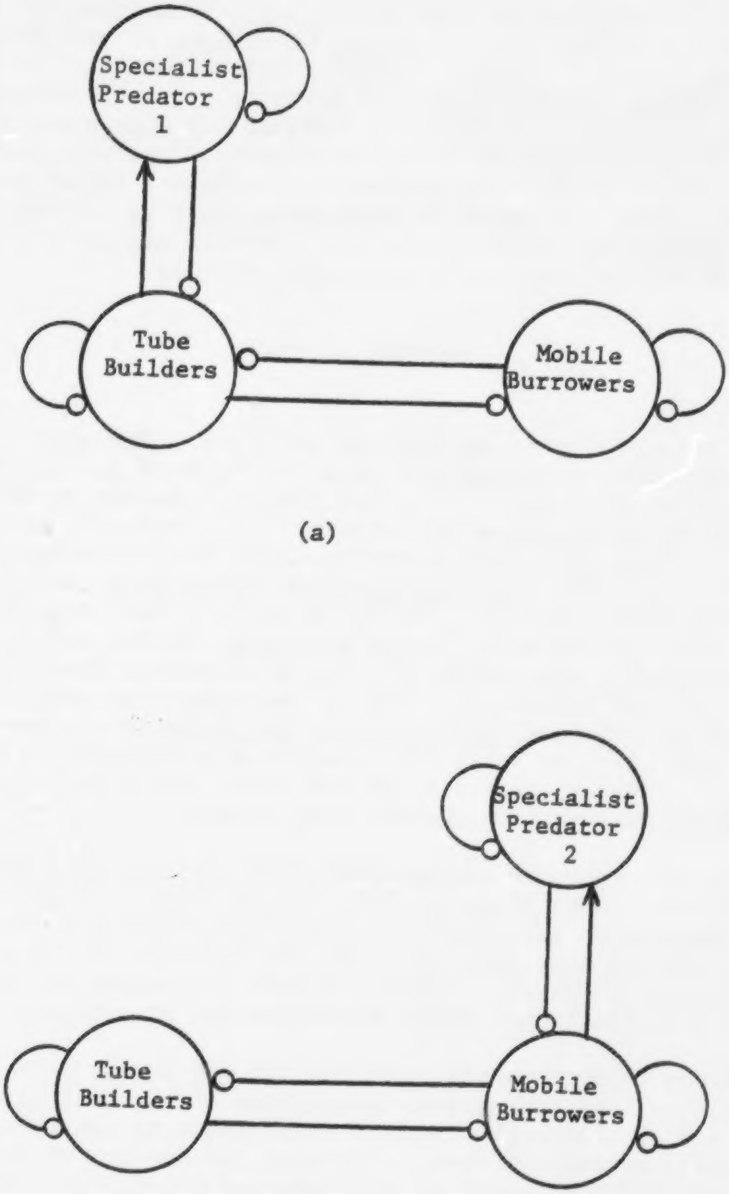
The six core models do not include predators. The major predators on the benthos include fish, epifaunal crustacea and some of the benthic infauna. Benthic predators, especially fish, are commonly assumed to be generalists because they can and sometimes do eat most taxa. However, in terms of effecting populations, the generalist predator among our functional groups is unlikely. Winter flounder, Pseudopleuronectes americanus, have been shown to select size-specific prey (12). In St. Margaret's Bay, Nova Scotia, during the early spring, the amphipod Photis reinhardi and the polychaete, Micronephthys minuta, are abundant. Winter flounder, however, consume the amphipod but not the polychaete. During the summer, as amphipods decline and the smaller-sized ostracod Nurmanicythese leisderma increases, the flounder does not switch. Yet, by fall, the flounder does consume the dominant Micronephthys. Clearly, prey size is the chief selection criteria except in very low abundances, and this changes over seasons.

Models 1 through 5 are reconsidered with specialized predators for each of the functional groups (Figures 7-11; Tables 9-13). In all cases, the central predictions on tube-builders/mobile-burrowers does not change. In other words, the predator does not alter the "network dynamics" for these two functional groups. However, predators effect themselves and filter-feeders. The specific prey functional group determines the direction of effect.

Concordance in model predictions for all six models is reassuring. Yet, it also indicates that the general description of the benthic community through the set of alternative network connections is inadequate for distinguishing between benthic patches. Important information about benthic ecology is missing, and the nature of this information will be discussed in the next section.

DISCUSSION

Mobile-burrowers, tube-builders, and filter-feeders are functional groups which lump species either in competition with each other or having a negative effect on at least one other group. Lumping goes beyond taxonomic similarity.


Specialist
Predator
1
Tube
Builders
Mobile
Burrowers
Specialist
Predator
2
Tube
Builders
Mobile
Burrowers

(a)

(b)

Figure 7. Model 7, which has model 1 as the core and specialist predator on (a) the tube-builder or (b) mobile-burrower.

TABLE 9. MODEL 7a,b PREDICTIONS ON DIRECTION OF CHANGE IN ABUNDANCE THROUGH CHANGE IN RATE OF GROWTH. SEE TABLE 3 FOR EXPLANATION OF PARENTHESIS SIGNS AND SUBSCRIPTS

	Filter Feeders	Tube Builders	Specialist Predators SP 1 SP 2
Tube Builders	+ +	- -	+ -
Mobile Burrowers	- -	+ +	- +
Specialist Predators SP 1 / SP 2	- +	+ -	- -

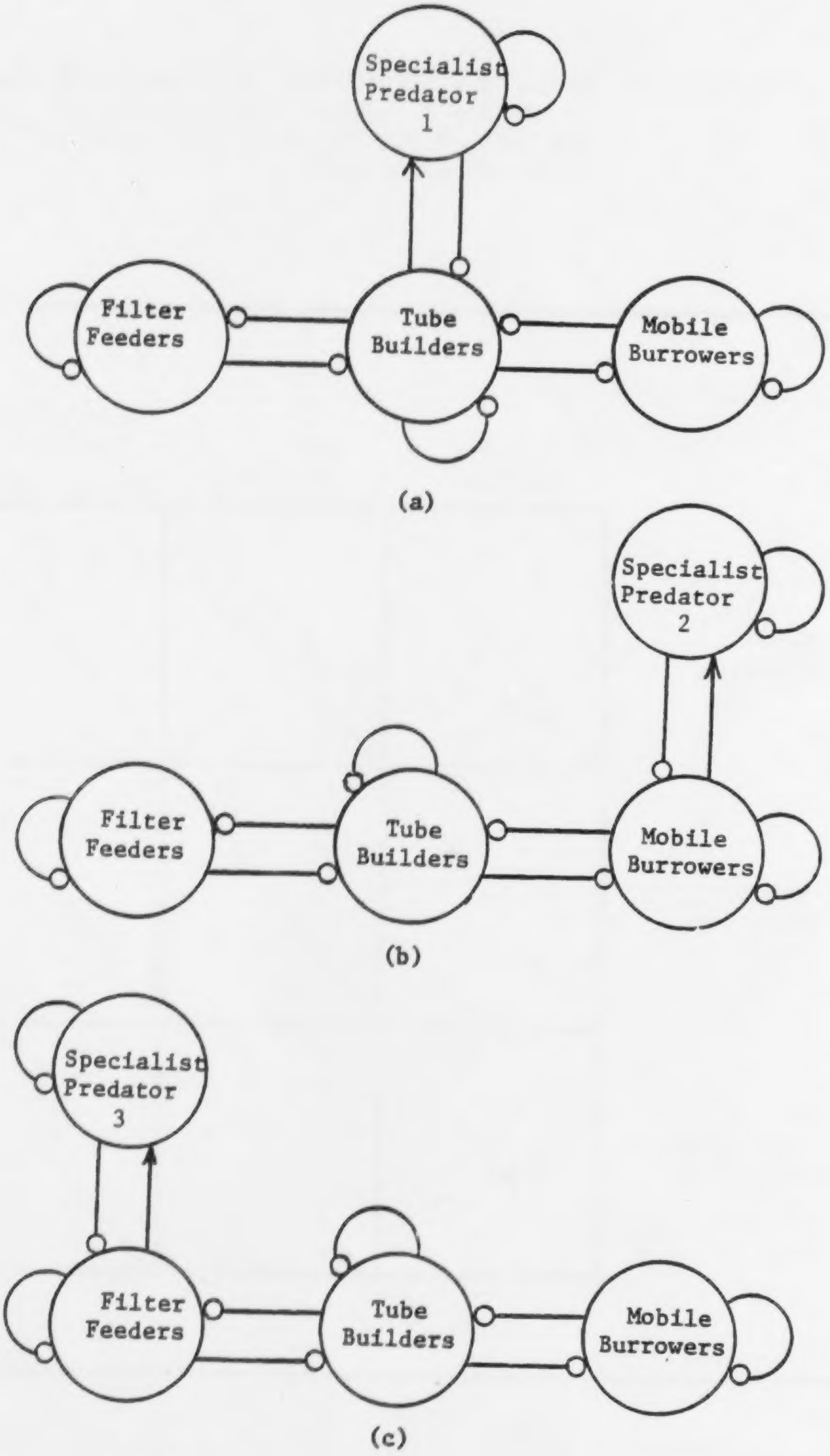

Specialist Predator 1
Filter Feeders
Tube Builders
Mobile Burrowers
(a)
Specialist Predator 2
Filter Feeders
Tube Builders
Mobile Burrowers
(b)
Specialist Predator 3
Filter Feeders
Tube Builders
Mobile Burrowers
(c)

Figure 8. Model 8, which has model 2 as the core and specialist predator on (a) the tube-builders, (b) the mobile-burrowers and (c) filter-feeders.

TABLE 10. MODEL 8a,b,c PREDICTIONS ON DIRECTION OF CHANGE IN ABUNDANCE THROUGH CHANGE IN RATE OF GROWTH. SEE TABLE 3 CAPTION FOR EXPLANATION OF PARENTHESIS SIGNS AND SUBSCRIPTS

	Filter Feeders	Tube Builders	Mobile Burrowers	Specialist Predators SP 1/SP2/SP3
Filter Feeders	? ? $(-)_1$	- - -	+ + +	- + $(-)_1$
Tube Builders	- - -	+ + +	- - -	+ - -
Mobile Burrowers	+ + +	- - -	$(+)_2$ $(+)_2$ $(+)_2$	- $(+)_2$ +
Specialist Predators SP1/SP2/SP3	+ - $(+)_1$	- + +	+ $(-)_2$ -	$(+)_c$ $(+)_c$ $(+)_c$

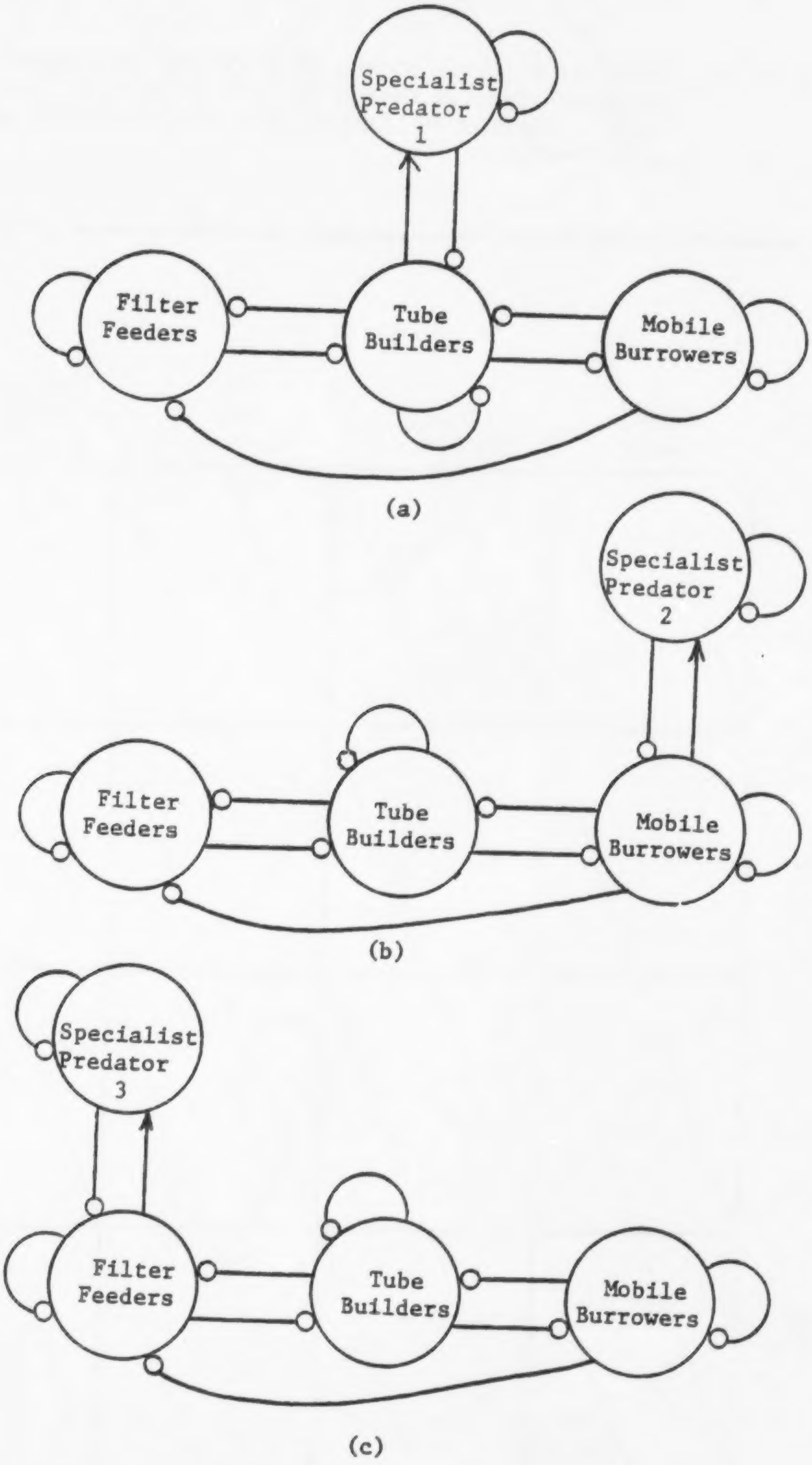

Specialist Predator 1
Filter Feeders
Tube Builders
Mobile Burrowers
(a)
Specialist Predator 2
Filter Feeders
Tube Builders
Mobile Burrowers
(b)
Specialist Predator 3
Filter Feeders
Tube Builders
Mobile Burrowers
(c)

Figure 9. Model 9, which has model 3 as the core and specialist predator on (a) the tube-builders, (b) the mobile-burrowers and (c) filter-feeders.

TABLE 11. MODEL 9a,b,c PREDICTIONS ON DIRECTION OF CHANGE IN ABUNDANCE THROUGH CHANGE IN RATE OF GROWTH. SEE TABLE 3 CAPTION FOR EXPLANATION OF PARENTHESIS SIGNS AND SUBSCRIPTS

	Filter Feeders	Tube Builders	Mobile Burrowers	Specialist Predators SP1/SP2/SP3
Filter Feeders	? ? $(-)_1$	- - -	+ + +	- + $(-)_1$
Tube Builders	(?)* (?)* (?)*	+ + +	- - -	+ - (?)*
Mobile Burrowers	(?)* (?)* (+)*	(-)* (-)* (-)*	$(+)_2$ (+) $(+)_2$	(-)* $(+)_2$ $(?)_2$
Specialist Predators SP1/SP2/SP3	(?)* ^(?)* $(+)_1$	- (+)* +	+ $(-)_2$ -	$(+)_c$ $(+)_c$ $(+)_c$

* Forced to negative

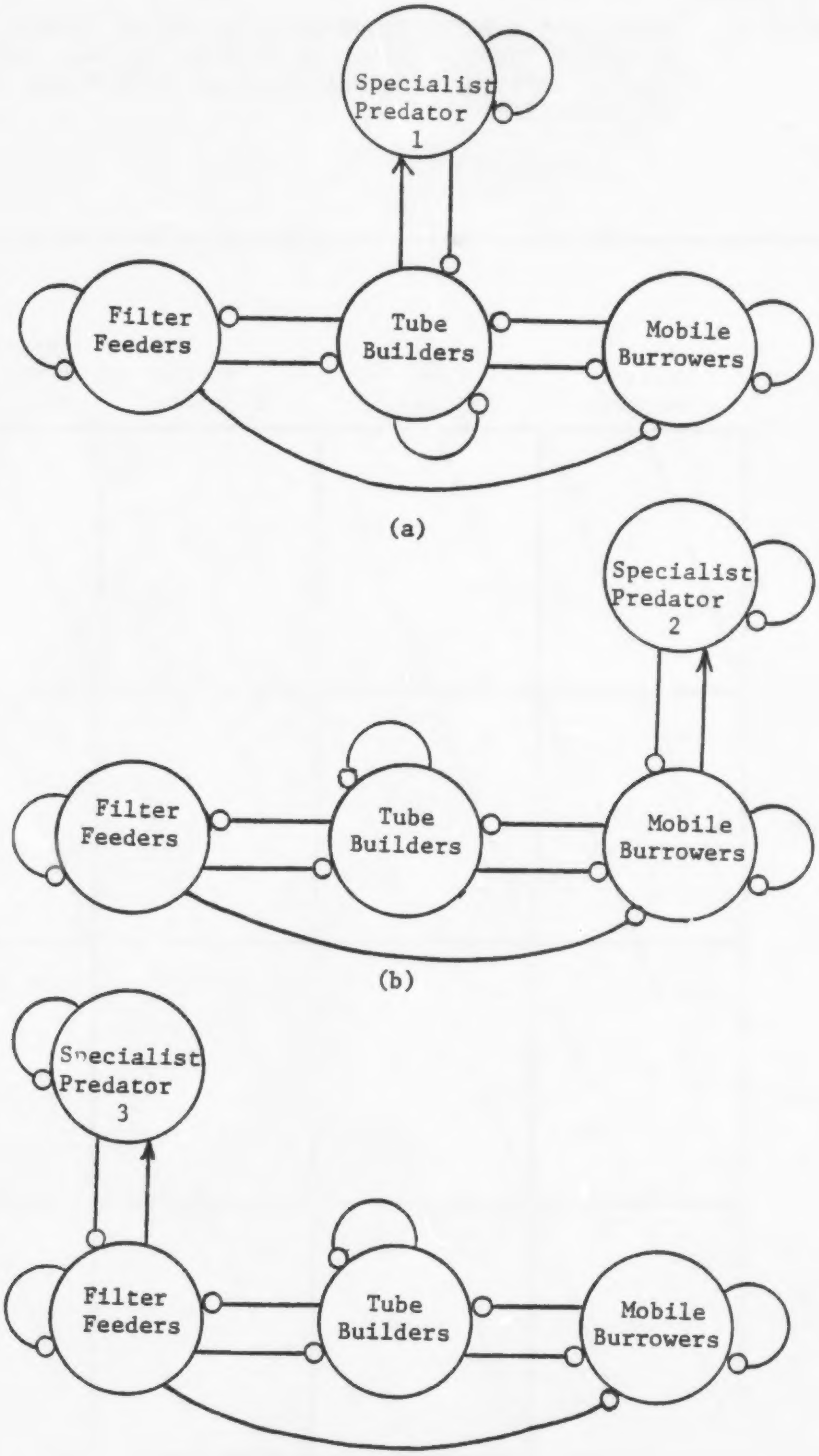

Specialist
Predator
1
Filter
Feeders
Tube
Builders
Mobile
Burrowers
(a)
Specialist
Predator
2
Filter
Feeders
Tube
Builders
Mobile
Burrowers
(b)
Specialist
Predator
3
Filter
Feeders
Tube
Builders
Mobile
Burrowers
(c)

Figure 10. Model 10, which has model 4 as the core and specialist predator on (a) the tube-builder, (b) the mobile-burrower and (c) filter-feeder.

TABLE 12. MODEL 10a,b,c PREDICTIONS ON DIRECTION OF CHANGE IN ABUNDANCE THROUGH CHANGE IN RATE OF GROWTH. SEE TABLE 3 CAPTION FOR EXPLANATION OF PARENTHESIS SIGNS AND SUBSCRIPTS

	Filter Feeders	Tube Builders	Mobile Burrowers	Specialist Predators SP 1/SP2/SP3
Filter Feeders	? ? $(-)_1$	$(+)_5$ $(+)_5$ $(+)_5$	$(+)_5$ $(+)_5$ $(-)_1$	$(+)_5$ $(+)_5$ $(-)_1$
Tube Builders	- - -	+ + +	$(-)_6$ $(-)_6$ $(-)_6$	+ $(-)_6$ -
Mobile Burrowers	+ + +	- - -	$(+)_2$ $(+)_2$ $(+)_2$	- $(+)_2$ +
Specialist Predators SP1/SP2/SP3	+ - $(+)_1$	- + $(-)_5$	$(+)_6$ $(-)_2$ -	$(+)_c$ $(+)_c$ $(+)_c$

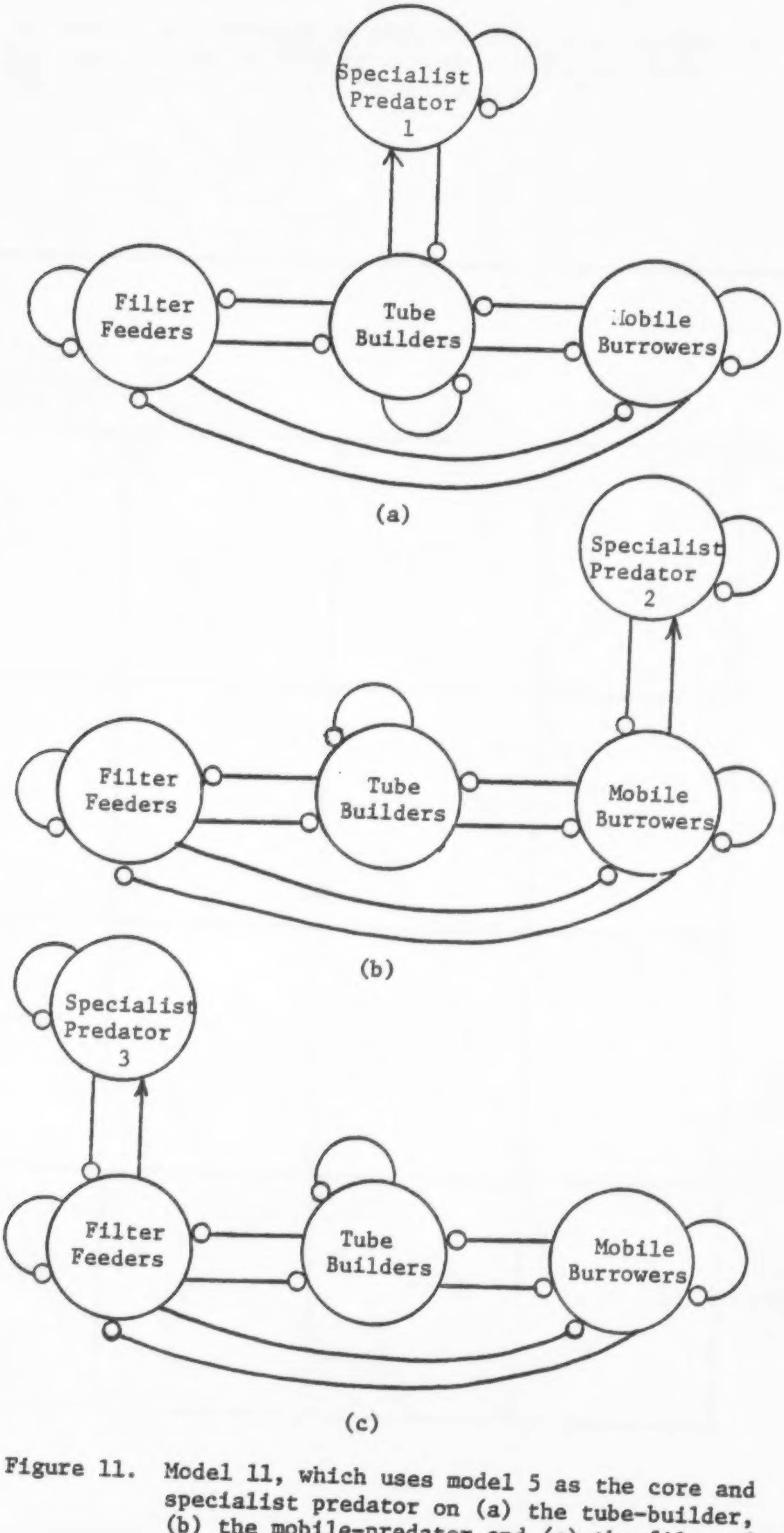

Specialist Predator 1
Filter Feeders
Tube Builders
Mobile Burrowers
(a)
Specialist Predator 2
Filter Feeders
Tube Builders
Mobile Burrowers
(b)
Specialist Predator 3
Filter Feeders
Tube Builders
Mobile Burrowers
(c)

Figure 11. Model 11, which uses model 5 as the core and specialist predator on (a) the tube-builder, (b) the mobile-predator and (c) the filter-feeder.

TABLE 13. MODEL 11a,b,c PREDICTIONS ON DIRECTION OF CHANGE IN ABUNDANCE THROUGH CHANGE IN RATE OF GROWTH. SEE TABLE 3 CAPTION FOR EXPLANATION OF PARENTHESIS SIGNS AND SUBSCRIPTS

	Filter Feeders	Tube Builders	Mobile Burrowers	Specialist Predators SP 1 SP 2 SP 3
Filter Feeders	? ? (-)$_{5,*}$	(+)$_{5}$ (+)$_{5,11}$ (+)$_{5,11}$	(+)$_{5}$ (+)$_{5,11}$ (-)$_{1}$	(+)$_{5}$ (+)$_{5,11}$ (-)$_{1}$
Tube Builders	(?)* (?)* (?)*	(-)$_{7}$ (?)$_{*}$ (?)* (-)$_{5}$ (?)*	(-)$_{6,*}$ (-)$_{6,*}$ (-)$_{6,4}$	(-)$_{7}$ (?)$_{*}$ (-)$_{6,*}$ (?)*
Mobile Burrowers	(?)* (?)* (?)*	(-)* (-)* (-)*	(+)$_{2}$ (+)$_{2}$ (+)$_{2}$	(-)* (+)$_{2}$ (?)*
Specialist Predators SP1/SP2/SP3	(?)* $(\hat{?})$* (+)$_{1}$	(+)$_{5}$ (?)* (+)* (-)$_{5,*}$	(+)$_{6,*}$ (-)$_{2}$ -	(+)$_{c,*}$ (+)$_{c,*}$ (+)$_{c,*}$

The burrowing polychaete Nephtys incisa is grouped with the small mobile-burrowing bivalve Nucula proxima.

Scale of applicability can change a model and results. Local patches will receive recruitment of settling larvae from a pool of larvae that are produced by adults outside the patch. In a large estuary or enclosed limited-interchange bay, recruitment is from larvae produced by adults within the bounds of the region. In the latter case, self-damping may be removed or diminished in magnitude. When strong self-damping is scale-dependent and necessary for stability as in the filter-feeder/tube-builder subsystem, then increasing scale from a patch to the bay can bring on subsystem instability. This not only effects the subsystem but changes the effect of parameter inputs to other components of the ecosystem.

Seasonal variation in predator selection, settling of larvae, and strength of composition can effect environmental assessment. For a specific time of year, different models may apply. A mud patch in spring has winter flounder consuming the tube-building amphipod, Photis. Any input to winter flounder during this time will cause an increase in mobile-burrowers and decrease in tube-builders. The same mud patch in fall has winter flounder switching prey, consuming the burrowing polychaete, Micronepthys. During this season, an input to winter flounder increases tube-builders and decreases mobile-burrowers. Thus, an assessment of benthic patches that receive impacts through winter flounder requires seasonally adjusted models.

Benthic impact sources are not always known, but they may be identified through the use of a series of benthic models. For example, data showing a positive correlation between mobile-builders and a fish predator corresponds to predictions of Model 3b; hence, the impact is a substance or activity that only effects tube-builder predators. When an impact source is known, then using the summary of predictions, we compare models with seasons.

Environmental assessment of benthic impacts requires two types of considerations. One is knowing which functional group is affected by the impact. Weathered No. 2 fuel oil increases mortality on all benthic infauna (13) and resulting analysis adds all three rows in a table of predictions.

Another consequence of an impact to the benthos is that the community dynamics are changed. Evidence from the Florida oil spill in Buzzard's Bay, Massachusetts suggests organisms are inhibited from using the most economical biochemical pathways (13). The fiddler crab, Uca pugnax can molt, show breeding coloration out of season and burrow too shallow for winter survival; the small estuarine fish, Fundulus heteroclitus, shows a lower rate of hepatic lipogensis and, in general, oil induced physiological stress increases demand on stored energy; petroleum contaminated sediments show the clam, Mya arenaria, and two species of mussels have reduced carbon flux.

We can extend the above hypothesis to consider for illustrative purposes that the tube-building amphipod, Ampelisca, through non-lethal concentrations of oil contaminated sediments, will impair tube construction or build tubes of reduced structural integrity. However, since it is the tubes of Ampelisca that interfere with mobile-burrowers, then reduced tube density or construction strength will effectively weaken the competitive link of Ampelisca on the mobile-burrowers. This reverses the previous assumption that the subsystem of tube-builders/mobile burrowers is unstable and consequently reverses many of the predictions. It is not sufficient to identify the organisms receiving the direct impact of human activity. There also must be some knowledge of how the impact effects the community strengths of interactions.

In summary, environmental assessment of the benthos requires data that goes beyond distribution and abundance of organisms. Specific site location and sediment characteristics will determine interconnections between species functional groups. Relative quantification through experimentation is necessary for the links between tube builders to filter-feeders compared with the link from mobile-burrowers to filter-feeders. Seasonal characteristics of predators and the selection of prey must be obtained or deduced from correlation data and the models. Finally, an estimate of source of settling larvae, whether local or global relative to the impact site, must be understood. The general benthic models presented here are to a first approximation accurate descriptors of the known or currently accepted hypothesis of benthic organism interactions. Understanding these models is perhaps a first guide to detailed data collection and constructing site specific models.

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Comments on
Qualitative Analysis in the
Setting of East Coast Marine Benthos

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One of the most pressing problems in contemporary environmental biology is the need to develop reliable approaches, firmly based in ecological theory, for predicting and thereby mitigating human impacts on natural ecosystems. In spite of this pressing need, most current approaches in "environmental toxicology" focus on responses of intact environmental systems. Although toxicological research on less-than-whole ecosystems is important and should be intensified, its results cannot be extrapolated to natural ecosystems in the field. The constraints imposed on species or species groups by their coupling within networks of matter and energy flow in ecosystems alter their dynamic behaviors in ways which are neither intuitively obvious nor predictable a priori. Thus, reliable impact assessment for natural ecosystems requires research focused directly at the system level rather than on components or subsystems of ecosystems.

Among the various approaches presently employed by environmental biologists to study ecosystem dynamics in natural and perturbed states, the qualitative technique known as loop analysis—originally developed by Richard Levins and explicated in the present paper by Puccia and Bennett (as well as in the following paper by Lane)—offers many advantages. This approach, conceptually similar to the earlier work of Mason on signal flow graphs, but more general and motivated by ecological concerns, derives its theoretical basis from the theory of differential equations, as represented near equilibrium points by matrices and associated linear graphs. The method focuses on the information that can be gained only from knowledge of the directions and signs of interactions among components of ecological networks. Thus, the approach avoids the many frustrations and reductionist aspects of quantifying the plethora of coefficients and input functions involved in large-scale ecosystem simulation models. More importantly, loop analysis can focus attention on critical connections or pathways in ecosystems, and thereby provide important direction to ecosystem research programs. In this sense, loop analyses may eventually form an integral step in the total development of more quantitative simulation models. Moreover, current uses of loop analysis are firmly grounded in ecological theory, at least at the level of the biotic community if not at the ecosystem level. Analyses of loop models allow one to investigate the effects of network topological structure on system dynamics and stability independently of the compositional details of that structure. This is perhaps the major advantage of the approach for both ecological theory and management application.

The present paper by Puccia and Bennett nicely summarizes the basis of loop analysis and illustrates its use in investigating qualitative changes in benthic marine communities found along the Atlantic Coast of North America to altered parameter inputs. Discussions in the paper clearly demonstrate how current understandings of interactions among functional groupings of benthic organisms can be translated into loop models having wide geographic applicability. The authors further demonstrate how the models can be manipulated to generate hypotheses or predictions concerning both correlations among state variables and changes in standing crops as a consequence of altered parameter inputs to the model. In a very nice twist, alternative structural models are studied in the hope of deriving "robust" results which do not depend on any one specific model structure. This approach should be taken more often; too much of "systems ecology" has generated predictions which are more strongly related to the properties of the mathematical formulation employed in a given model than to the properties of the ecological system being modeled. Finally, the authors correctly stress the importance of scale and seasonal effects to their model analyses. (However, it is not clear how loop models offer advantages over other types of ecological models in investigating scale-dependent or seasonal effects on model results.)

In spite of the many advantages inherent in loop analysis, it remains a relatively new approach to studying ecosystems. Thus, problems in its application remain to be resolved. Also, some reservations exist concerning the specific way in which loop analysis is used in this paper. In the remainder of my response I will briefly summarize several of these problems-reservations. It should be emphasized, however, that research described by Puccia and Bennett is preliminary and in progress, so that my comments should not be taken as any final criticism of the loop model approach. Many of these points may be resolved in subsequent work. Indeed, most of these issues are ones that require additional research for their resolution.

First, in spite of comments in the paper to the contrary, the models developed here are not models of ecosystems, but rather of benthic invertebrate communities. The benthic community is treated as though it were isolatable from the remainder of the ecosystem network structure. Effects of other biotic and physicochemical variables are assumed to enter only as so-called "parameter inputs" to the benthic network structure. In other words, the remainder of the ecosystem is treated as forming the separate (and separable) environment of the benthic community. I would suggest that this is a flawed conceptual approach, that the benthic community is not isolatable in the above sense, and the meaningful ecosystem analysis and impact prediction require that ecosystems be treated as intact, functional, biogeochemical systems rather than as discussion on illustration of the use of loop analyses for studying benthic communities. The loop analysis approach also have promise for assessing human impacts on natural ecosystems. However, for the reasons summarized above (and others), the ability to use loop analysis in routine environmental impact assessment remains to be demonstrated conclusively.

networks of only select biotic state variables. Note that this is a criticism of the approach taken here rather than of loop analysis per se.

A second problem concerns the need for what might be called ad hoc hypotheses to resolve ambiguities in loop model predictions of changes in benthic standing crops following altered parameter inputs (e.g., see Tables 2-6 and Appendix II). The use of such hypotheses is certainly an integral part of the research process, and forms a vital link between the conceptual model of a given system and experimentation on that system. However, the need for such hypotheses may make loop analysis only a research tool, limiting its utility in routine impact assessment. Moreover, many of the hypotheses employed by Puccia and Bennett seem a bit tentative and require verification or further justification before results based upon them can be accepted unequivocally.

A third problem involves the dichotomy between the direction and magnitude of predicted change in an ecological state variable. Loop analyses can reveal whether a given state variable will increase, decrease, or remain unchanged following a specific change in some parameter input. Yet it cannot reveal whether a variable will increase by 10 percent or 1000 percent. Such information, however, is vital in both theoretical ecology and impact assessment. An increase of 2 percent in some state variable might be an insignificant change in a given system, whereas a change of 200 percent would probably be highly significant. Clearly, we need to be able to discriminate between such cases.

Fourth, since loop analysis is a near-equilibrium technique, it is questionable whether it can provide needed information to make reliable impact assessments. I do not mean to criticize the local equilibrium assumption which is inherent in loop analysis; it has been an exceedingly powerful approach in many areas of science. However, human impacts on ecosystems are qualitatively and quantitatively different from "natural" dynamics in ecological processes at various space-time scales. Human interventions can alter entire network structures, and push ecosystems into entirely new and perhaps unanticipated basins of attraction. Whether loop analysis can provide useful predictions in such cases is a question requiring additional attention.

Finally, most (though not all; see following paper) of the work on loop analysis to date has been theoretical. Additional work on the validation or verification of predictions of loop models is needed before the approach gains wide use. Such work should focus not only on actually performing experiments to validate loop model predictions, but also in developing appropriate methods for validating such predictions in a rigorous sense. Validation of loop models is likely to be quite different from validation of quantitative simulation models.

In summary, it is probably fair to conclude that loop analysis remains a valuable research tool for investigating effects of ecosystem structure on functional dynamics. The present paper provides a stimulating

USING QUALITATIVE ANALYSIS TO UNDERSTAND PERTURBATIONS TO MARINE ECOSYSTEMS IN THE FIELD AND LABORATORY

by

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ABSTRACT

Qualitative analysis is underutilized in many areas of science. This study involves an application of one qualitative approach (loop analysis) to a marine ecosystem that is undergoing perturbation. In loop analysis, signed diagraphs are used to represent a complex system and variables are connected based on the sign of their interaction coefficients. The study system is Bedford Basin, Nova Scotia, which is a small semi-enclosed marine basin. Analysis of the field community showed that there is a high level of organization that is trackable over the annual cycle. One loop diagram could be used to explain much of the observed correlation patterns among the ecological variables that were measured. The basic ecological structure consists of a two layered system with nine functional groups (two nutrients, two phytoplankton and five zooplankton variables). The parameter input to the network enters low in the trophic hierarchy through the nitrogen complex.

The field loop diagram was studied by establishing two laboratory communities in a large divided tank. One side of the tank was enriched with nitrogen, phosporus and silica. The enriched side of the tank exhibited higher levels of biomass for most groups of organisms than the unenriched side. The loop diagram was similar for both sides of the tank and it differed in five ways from the field diagram. There were two time periods in which the plankton dynamics differed in both sides of the tank. This temporal sequence was similar to those observed in the field for spring bloom conditions. Interestingly, the major parameter input entered at the algal level and not at nutrients in the first part of the experiment. During Time 2, parameter inputs occurred through silica for the enriched side and through flagellates for the unenriched side of the tank.

Loop analysis is a useful approach for analyzing ecological complexity which is needed for meaningful environmental impact assessment.

INTRODUCTION

At present, a satisfactory method of anticipating the impact of multiple human interventions in ecosystems does not exist. Ecological complexity is staggering. Coastal marine ecosystems may include several hundred variables (biotic and abiotic) which are interconnected in thousands of feedback pathways. Each pairwise interaction can result in +, - or 0 change in the rate of increase of each variable. Thus, for a ten species ecosystem there are 100 pairwise interaction coefficients (10^2) and 3^{100} patterns of ecosystem interaction. Even though many of these patterns are not ecologically plausible, the number of possible networks is much greater than that which could be systematically studied and experimentally verified. May (1) conducted a computer study of stability and complexity in randomly-generated ecological networks; he found there were billions or googols (10^{100}) of biologically reasonable systems. Lawlor (2) demonstrated that for 40 species, there would be 10^{764} networks of which 10^{500} would be biologically reasonable. They would be so sparse that random sampling would never find any of them. Lawlor (2) further estimated that for a 20 species network there is a 95% expectation of never encountering an ecological system in ten years if 1 million hypothetical networks were generated per second by computer.

In addition to delineating a feasible network, there is the problem of determining the major entry points of the perturbation (for example, an oil spill) into the network and then to predict the impact of this stress on individual components as well as on the total network. Even in relatively simple systems (and few ecosystems are simple) there can be many complex pathways of effect between an impact (parameter input) and a particular variable. Often direct effects, that are traditionally measured in controlled laboratory experiments involving isolated sub-systems, are nullified or reversed when they occur in ecological networks (3). For example, pesticides kill insects directly in laboratory containers but rarely control these pests in nature.

To make accurate predictions for environmental impact assessments the multiple causal pathways that exist between the stress and its effects on ecosystem components need to be understood for realistic ecological networks. The relationship between correlation and causality has been an uneasy one in ecological research. Although significant correlations may be found between pairs of ecosystem variables, at best, ecologists can only infer causal mechanisms and often unknowingly, nonsense correlations are supported by their inferences. In aquatic environments many types of parameters enter the ecological network in different places. For example, temperature and light are natural parameter inputs. Humans affect ecosystems in many other ways: thermal pollution, toxic chemicals, physical alterations, oil spills and natural parameter inputs are interrelated with these human interventions. Several current controversies center on the basic question of whether the main parameter enters above (vertebrate predation) or below (nutrient enrichment) in the aquatic trophic heirachy. Thus, given that aquatic ecosystems are always partially-specified, it is not surprising that correlation patterns of aquatic variables and more importantly, the causal mechanisms underlying these patterns, have not been rigorously delineated.

Much of current ecosystem-level research relies on large-scale quantification efforts. Often masses of data are interpreted using computer simulation models which are based on complicated differential equations used to represent the system. Precise values of parameters are usually required. Qualitative methodology departs markedly from those quantitative approaches in that it is mainly concerned with delineating the qualitative structure of complex networks without formulating the equations. Quantification is used to resolve ambiguity and to gain further insight. The qualitative approach is useful for understanding ecosystem complexity and in particular, cause-effect relationships in perturbed ecosystems. While quantification is often desirable, the initial use of qualitative analysis enables the investigator to be more selective in subsequent quantification efforts.

The main objective of the present study is to develop a qualitative methodology for application to environmental impact assessment problems. Qualitative networks of several coastal marine communities from the Davis Strait in Northern Canadian waters to the Chesapeake Bay bordered by Maryland and Virginia are being developed and studies. This paper reports on one example of using a qualitative technique (loop analysis) to understand the network of a plankton community both in the field and laboratory. Microcosm and large enclosure experiments involving whole communities have proved useful in aquatic ecology since they can be used to integrate field observation with laboratory experimentation (4,5,6,7,8). Loop analysis is applied here to determine if the laboratory network is similar to its natural counterpart and to test loop analysis predictions in a semi-controlled situation.

METHODS

BEDFORD BASIN

Study Area

The study was conducted in Bedford Basin, Nova Scotia, which is a semi-enclosed marine basin separated from the Atlantic Ocean by a channel, Halifax Harbour, 10 km long, 20 m deep, and 400 m wide at the narrowest point. The basin is 5 km long with a maximum depth of 72 m and a surface area of 17 km^2. Freshwater input from the Sackville River varies between 10^4 and 10^7 m^3/d. The tides are of a semi-diurnal type, 0.6-2.0 m in amplitude. The Basin has been subject to considerable stress from human intervention (9, 10) and is prone to anoxia. There have been many studies conducted on Bedford Basin because of the proximity of scientists at the Bedford Institute of Oceanography and Dalhousie University including basic oceanography, nutrient-phytoplankton-detritus relationships (11-17) and zooplankton dynamics (18-24).

Field Sampling and Laboratory Processing

All samples and measurements were taken from a fixed navigational buoy at approximately six week intervals from May, 1974 to March, 1975, on seven 24 hour cruises. Plankton, particulate organic carbon and nitrogen samples were collected from eight depths (0, 5, 10, 15, 20, 25, 40 and 55 m) four to six times per 24 hour period. (Four to six temperature, salinity, sigma T, oxygen, primary production and chlorophyll profiles were determined for each cruise, but these data are not discussed here).

Triplicate one-liter samples were drawn from van Dorn bottles at the eight sampling depths for P.O.C. and P.O.N. analysis. The water was filtered with a 191 mμ mesh prior to vacuum filtration onto 0.8 mμ silver membrane filters and sodium sulfate was added to the sample to remove inorganic salts. Immediately after filtration, the samples were placed over dry ice, and stored in a freezer until analysis took place. Carbon and nitrogen values were obtained using a Hewlett Packard model 185 CHN analyzer. These nutrients were collected at the eight depths with van Dorn water samplers. 75 ml of water were preserved in 25 ml of Transeau's solution and a 5 or 25 ml aliquot (depending on phytoplankton abundance) was enumerated using an inverted microscope. The counts were then converted to cell numbers/m^3. Phytoplankton abundances were combined into the following groups: small diatoms (0-40 mμ), large diatoms (> 40 mμ), small flagellates (> 40 mμ), and _Ceratium_ spp. The categories used were A_1 for the two size classes of diatoms and A_2 for the flagellates.

Zooplankton were collected by ten minute horizontal tows of two calibrated Clarke-Bumpus plankton samplers outfitted with No. 10 (158 mμ) nets. The samplers had a 12.7 cm diameter. Generally, six complete profiles were sampled during the 24 hour period of each cruise. Samples were concentrated and preserved in a 4% formalin solution buffered by hexamethylenetetramine. (The smaller forms were not adequately sampled and are therefore not included in this report). Cladocerans and copepods were enumerated by counting at least two sub-samples, representing .03% of the total sample. A Wild M-7 dissecting microscope was used. Large forms, _Calanus finmarchicus_ and _Sagitta elegans_, were enumerated in total. The latter species was divided into two groups: small (less than 5 mm) and large (more than 5 mm). Nine other species were identified. Copepodites, nauplii, _Centropages_ spp, and _Acartia_ spp, were not enumerated to species. There were sixteen species or groups used in this study with copepods, cladocerans and small _S. elegans_ grouped in the Z_2 and Z_3 categories and adult _S. elegans_ and _C. finmarchicus_ grouped in the S category.

TOWER TANK

The tower tank is a single cylinder, 10.5 m deep and 3.7 m wide, containing over 100 m^3 of water; it is a key component of the Dalhousie University Aquatron Facility. The tank and its design are described by Balch _et al_. (25). A divider was installed so that control and experimental treatments could be run simultaneously. In this study thermal and salinity stratifications developed within the water column.

In the nutrient enrichment experiment, side A (Tower A) was enriched at approximately 2X ambient concentrations of total nitrogen, phosphorous, and silica as measured in nearby waters, while side B (Tower B) served as a control. The communities were collected in Bedford Basin and added (after careful mixing) in similar densities to Tower A and Tower B. It was not possible to obtain growth of _Sagitta elegans_, an arrowworm, which is a major invertebrate predator in Bedford Basin. Six depths were sampled weekly for six weeks. Animals were then monitored for several additional weeks.

Temperature and salinity were measured at each depth using a Beckman RS-5 temperature and salinity probe. A hose and pump arrangement were used to sample the water column, with the hose being attached to a specially-designed horizontal sampler which made it possible to sample several locations at one time. Approximately one minute was allowed for the hose to clear for each sample and then one 12 l and 3 one l bottles were filled along with a 1000 ml graduated cylinder. The sides were sampled alternately at each corresponding depth. 50 ml of the water collected at each depth was used for a nutrient sample which was later analyzed on the auto-analyzer for NO_3, PO_4, and SiO_3. PO_4 has been classified under N_1, NO_3 under N_2, and SiO_3 under Si.

500 ml of water was filtered through a 10mμ mesh, and the residue was then placed in 100 ml of Transeau's solution for phytoplankton enumeration. The phytoplankton were counted using two 25 ml setting chambers, and transects were counted on a Zeiss inverted microscope. Diatoms were grouped together and are referred to as A_1, while flagellates are grouped into A_2. A 500 ml sample was taken for chlorophyll, and 2-250 ml replicates were filtered on 45mμ Millipore filters and analyzed by fluorescence. The 12 l sample was filtered through a 40mμ mesh, and this residue was preserved in 4% formalin for zooplankton enumeration. The animals were counted on a Wild M-7 dissecting microscope. Cladocerans and copepods were enumerated by counting at least two sub-samples, while larger forms were enumerated in total. Polychaete larvae were put into the grouping M, and the rest of the zooplankton are grouped under Z_2 and Z_3. 15 species of zooplankton and 10 species of phytoplankton were identified. Microzooplankton (Z_1) were not counted.

LOOP ANALYSIS AND DATA CALCULATIONS

Loop analysis is a qualitative, network technique that uses signed diagraphs to represent sets of interacting variables. The mathematical formalism is described in detail elsewhere (26-28). Lane and Levins (3) have studied hypothetical freshwater plankton communities using this methodology and Briand and McCauley (29) conducted lake manipulation studies and tested loop analysis predictions with their results.

Figure 1 illustrates a four variable aquatic ecosystem. The large circles enclose the variables (N,A,H and C). The arrowheads indicate a positive effect on the variable the arrowhead touches and similarly the circleheads indicate a negative effect on the variable they touch. The signs (+ and -) represent the qualitative effects of one variable on the rate of change of the second variable. (Thus, the signs are equivalent to the partial derivatives of the equation of each variable's rate of change, as a function of all other variables, evaluated at equilibrium). For example, herbivores(H) consume algae(A) causing a (positive) increase in H and a (negative) decrease in A.

The following definitions and rules summarize loop analysis as it is used here:

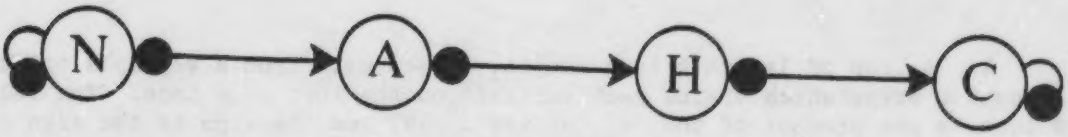

Figure 1. Loop diagram of a four variable aquatic ecosystem. Nutrient(N) and carnivore(C) variables are self-damped. Algal(A) and herbivore(H) variables are not self-damped. A circlehead indicates a negative effect on the adjacent variable and an arrowhead represents a positive effect on the adjacent variable. N-A, A-H, and H-C interactions represent predator-prey relationships. For example, carnivores(C) are increased by feeding on herbivores(H) whereas herbivores are decreased through carnivore predation.

TABLE 1. PREDICTIONS OF DIRECTED CHANGES IN STANDING CROPS FOR VARIABLES SHOWN IN FIGURE 1

Increase in: ▼	N	A	H	C	Variable →
N	+	+	+	+	
A	-	+	+	+	
H	+	-	+	+	
C	-	+	-	+	

TABLE 2. PREDICTED CORRELATIONS BETWEEN PAIRS OF VARIABLES SHOWN IN FIGURE 1 FOR INCREASES IN NUTRIENT(N) AND CARNIVORE(C)

Variable Pair ▼	Increase in: →	N	C
N-A		+	-
N-H		+	+
N-C		+	-
A-H		+	-
A-C		+	+
H-C		+	-

1. A loop of length k is a simple, closed path from a variable to itself through k steps which visits each variable on the loop only once. The value of a loop is the product of the α_{ij} of its links, and the sign is the sign of that product. A loop of length 0 is by convention positive and has the value +1. Feedback is defined as the effect of a variable on itself by way of intervening variables.

2. Mathematically, the feedback at level k, (F_k), in a system of $n > k$ variables is defined by $F_k = \Sigma\ (-1)^{m+1}L(m,k)$. Feedback at level k is summed over all sets of the products of m disjunct loops that total k elements. Disjunct loops have no variables in common (L = loops).

3. Loops of length 0 have a value of +1 and $F_0 = -1$. This is an algebraic convenience.

4. A path $P_{ij}^{(k)}$ is a product of $(k - 1)$ alpha values from X_j to X_i involving k variables, none of which are visited more than once. $P_{ii} = 1$.

5. The complement of a path is the set of variables not on the path.

6. Let C_h be any of s parameters of the system $dX_i/dt = f_i(X_1,X_2,X_3,\ldots, X_n; C_1,C_2,C_3 \ldots,C_s)$. Then the effect of a change in C_h on the equilibrium level of any variable $(\hat{X}_j)$ in the system is

$$\partial\hat{X}_j/\partial C_h = \Sigma\ (\partial f_i/\partial C_h) \times P_{ji}^{(k)} \times F_{n-k}[\mathrm{Comp}\ P_{ji}^{(k)}]/F_n,$$

that is, if C_h is a positive input to X_i; then its effect on X_j will have the sign of the sum of the products of each path from X_i to X_j, each multiplied by the feedback of its complement, and all divided by the feedback of the whole. The input along that path has no effect.

In Figure 1, the path from nutrient to carnivore is positive, whereas the path from carnivore to nutrient is negative. There are three loops of length two representing predator prey interactions and two self-damped loops (length one). There are no loops of higher length (level) that is, involving more than two variables. Table 1 gives the qualitative predictions of changes in standing crops for four parameter inputs (an increase in N, A, H and C) shown in the left-hand margin. The changes are read across each row. For example, an increase in A, perhaps reflecting a better environment for algae (more optimal temperature or light regime), results in a decrease in N and increases in A, H and C.

These directed changes can also be used to predict correlations between changes in pairs of variables (Table 2). For example, if N is increased then there will be positive correlations between the changes in standing crops of all variable pairs. If C is increased, only N-H and A-C pairs will be positively correlated and all other pairs will exhibit negative correlation. Both directed changes in standing crops and the resultant correlation values generated by loop analysis can be tested with field and laboratory data sets. In this study, the field data are used to study correlation predictions and the laboratory data are tested against directed change predictions.

The data correlations for the variables measured in the field cruises on Bedford Basin were generated as Pearson correlation coefficients using SPSS and the Dalhousie CDC 6400 computer. The loop analysis calculations were done by hand.

RESULTS

BEDFORD BASIN

A summary of the data correlations taken from a standard correlation matrix is given in Table 3. Most pairs of variables are strongly correlated and consistent within functional groups of organisms. (Functional groups are loosely-defined ecological units. They are used as variables in the loop diagrams and also to organize the data sets. The definitions are based on several criteria including: toxonomic affinities, feeding preferences and behaviors, chemical similarities, developmental stage, and size of organisms and their foods). The correlation values were generated over all data points for each pair of variables for the total year of sampling. Preliminary analysis of individual correlation matrices per date showed that they were consistent with the all-dates correlation matrix. These correlation patterns persist over the annual cycle despite high spatial and temporal variation. This indicates a well-organized community structure which is trackable. Only those data correlations that refer to variables that are included in the following (Bedford Basin) loop diagram, and can therefore be tested, are included in this summary.

For testing the loop predictions, the correlation values were grouped as negative at the 75 and 95% significance levels and grouped similarly for positive signs. In general, there was a lot of positive correlation which indicates that the major parameter input is entering low in the trophic web at the nutrient or algal levels. (See Figure 1 which shows how positive correlation is generated upwards in a food chain). There was little negative correlation exhibited in this data set.

The loop diagram representing the Bedford Basin ecosystem is illustrated in Figure 2. It contains 9 variables and the major parameter input enters at N_2, nitrogen complex. The network is essentially a two-layered system. The generation times of the organisms are effective in isolating subsystems in the diagram. The top layer of the diagram is more effectively isolated from the bottom layer during periods of thermal stratification. The standing crop predictions for Figure 2 are given in Table 4. The question marks indicate that there are both negative and positive pathways or complements that result in ambiguous (?) predictions. Z_1, microzooplankton, functions as a satellite variable; it changes little itself except when it receives a direct parameter input. The predicted correlation matrix for the Bedford Basin loop diagram is given in Table 5. Only the correlation signs for the predictions arising from a parameter input at N_2 are given here. Each parameter input to a loop diagram variable usually yields different correlation predictions. Also, one or two link changes in a loop diagram can result in marked changes in both the standing crop and correlation predictions.

TABLE 3. SUMMARY OF CORRELATION COEFFICIENTS FOR BEDFORD BASIN DATA SET*

Variables** ▼	+	(+)	0	(-)	-	Sum	Correlation sign ⟶
Z x Z	63	3	23	2	0	91	
Z x S	2	0	7	7	12	28	
Z x A_1	0	1	20	4	3	28	
Z x A_2	20	3	19	0	0	42	
Z x N	11	2	14	1	0	28	
S x S	1	0	0	0	0	1	
S x A_1	0	0	2	2	0	4	
S x A_2	0	0	2	1	3	6	
S x N	0	0	2	2	0	4	
A_1 x A_1	1	0	0	0	0	1	
A_1 x A_2	0	2	4	0	0	6	
A_1 x N	3	1	0	0	0	4	
A_2 x A_2	2	0	1	0	0	3	
A_2 x N	6	0	0	0	0	6	
N x N	1	0	0	0	0	1	
						253	

* + and - represent positive and negative correlation values significant at the 95% level and signs in parentheses indicate a 75% significance level.

** Key: Z = zooplankton, S = Sagitta - Calanus complex,
A_1 = diatoms, A_2 = dinoflagellates, and N = nutrients (nitrogen and carbon).

To test a loop diagram for correlation relationships, a table of correlation predictions versus data correlation values is developed (not shown). Of the 253 data correlation values given in Table 3, six represented self-correlation terms that could not be tested with loop analysis (S-S, A_1-A_1, A_2-A_2, N-N) Z-Z correlations could be tested because Z_2 and Z_3 are separate variables in loop diagram and they a predicted correlation value. 157 data correlation values were of the correct sign and 21 were of the wrong sign. 67 values were zeroes, that is, statistically insignificant when a + or - value was predicted by loop analysis. Almost all of the zero data correlation values were in the direction of the prediction. That a correlation is measured as zero in the field when loop analysis yields a + or - prediction does not necessarily indicate that the theoretical predictions are in error; it often means that the variables were not measured adequately. Only 8% of the data correlation values were predicted as definitely wrong by these loop analysis results.

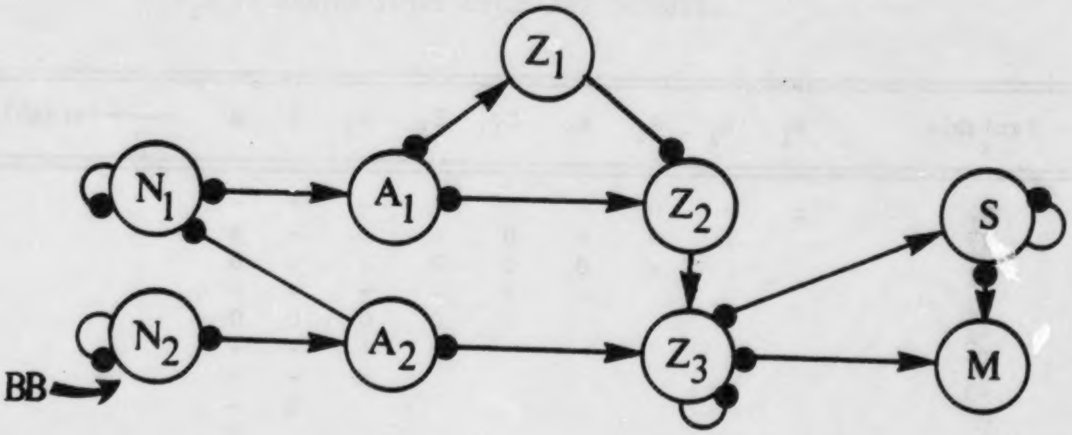

Figure 2. Loop diagram of Bedford Basin ecosystem. The network includes two nutrients complexes (N_1 = phosphate-silica complex and N_2 = nitrogen complex), two algal groups (A_1 = diatoms and A_2 = flagellates), and five zooplankton groups (Z_1 = microzooplankton, Z_2 and Z_3 = cladocerans and copepods, M = macrozooplankton and S = deep living adult Sagitta elegans and Calanus spp. group).

TABLE 4. PREDICTIONS FOR DIRECTED CHANGES IN STANDING CROPS FOR VARIABLES SHOWN IN FIGURE 2*

Increase in: ▼	N_1	N_2	A_1	A_2	Z_1	Z_2	Z_3	S	M	variable →
N_1	+	-	0	+	0	-	-	+	+	
N_2	-	+	0	+	0	+	+	-	+	
A_1	-	-	0	+	0	-	-	+	-	
A_2	-	-	0	+	0	+	+	-	+	
Z_1	-	-	-	+	+	-	-	+	-	
Z_2	+	+	0	-	?	+	+	-	+	
Z_3	+	+	0	-	0	-	+	-	+	
S	-	-	0	+	0	+	-	+	?	
M	?	?	0	?	0	?	?	-	+	

* See legend for Figure 2 for meaning of the symbols.

? = Ambiguous result.

TABLE 5. PREDICTIONS OF CORRELATION SIGNS FOR FIGURE 2. ASSUMING PARAMETER INPUT ENTERS AT N_2*

Variable ▼	N_1	N_2	A_1	A_2	Z_1	Z_2	Z_3	S	M	⟶ variable
N_1	x	-	0	-	0	-	-	+	-	
N_2		x	0	+	0	+	+	-	+	
X_1			x	0	0	0	0	0	0	
A_2				x	0	+	+	-	+	
Z_1					x	0	0	0	0	
Z_2						x	+	-	+	
Z_3							x	-	+	
S								x	-	
M									x	

* Key: x = Self correlation term could not be calculated, other symbols are given in Table 4.

TOWER TANK

The tower tank was used as an experimental tool for the following purposes: 1) to determine if loop diagrams could be constructed with laboratory data, 2) to compare these diagrams with the ones developed for the field data, 3) to delineate how nutrient enrichment affected the laboratory community, and 4) to evaluate if laboratory testing of loop analysis is feasible and useful. The data set in the tower tank experiment (6 dates x 6 depths = 36 samples) for each side is substantially different than its field counterpart.

Depth and Data Variability

Temperature, salinity, oxygen concentration and conductivity levels were similar in the tower tank on each date. Because of this similarity, only data from Tower A is shown (Figure 3). Temperature gradually shifted 1½ °C in the bottom water during the experiment. Heating from the overhead lights caused the surface water to warm more quickly and two thermal periods were defined (November 14 - November 28 and December 12-19). Oxygen concentration exhibited a more marked surface shift in the latter part of the experiment than did temperature. Undoubtedly, high abundances of phytoplankton at the surface contributed to these high oxygen concentrations.

Nutrients were added to Tower A as P-PO4 + N - NO_3 + Si-SiO_3 in the ratio 1:5:8 on November 11, 24, and December 12 and 19. Their concentrations changed markedly with depth throughout the experiment although depth distributions were similar in the two sides of the tank for each date (Figure 4). There was a substantial decrease in nutrients in the middle portion of the water column on December 5 and 12, followed by a decrease in the surface water on December 19. Nutrients ratios in Tower B remained more consistent than they did in Tower A. The mean values over dates of all nutrients were higher in Tower A than in Tower B, however, on particular dates some nutrient concentrations were higher in Tower B.

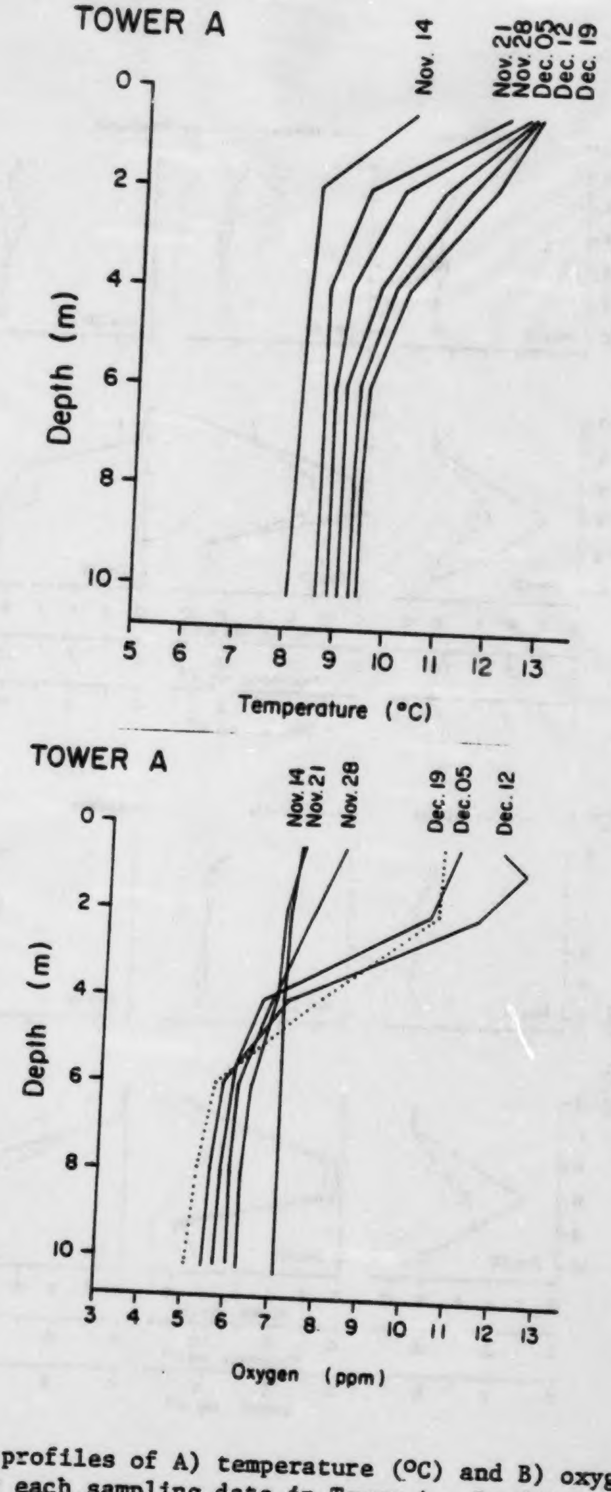

Figure 3. Vertical profiles of A) temperature (^{o}C) and B) oxygen concentration (ppm) for each sampling date in Tower A. Profiles of the same factors in Tower B were similar.

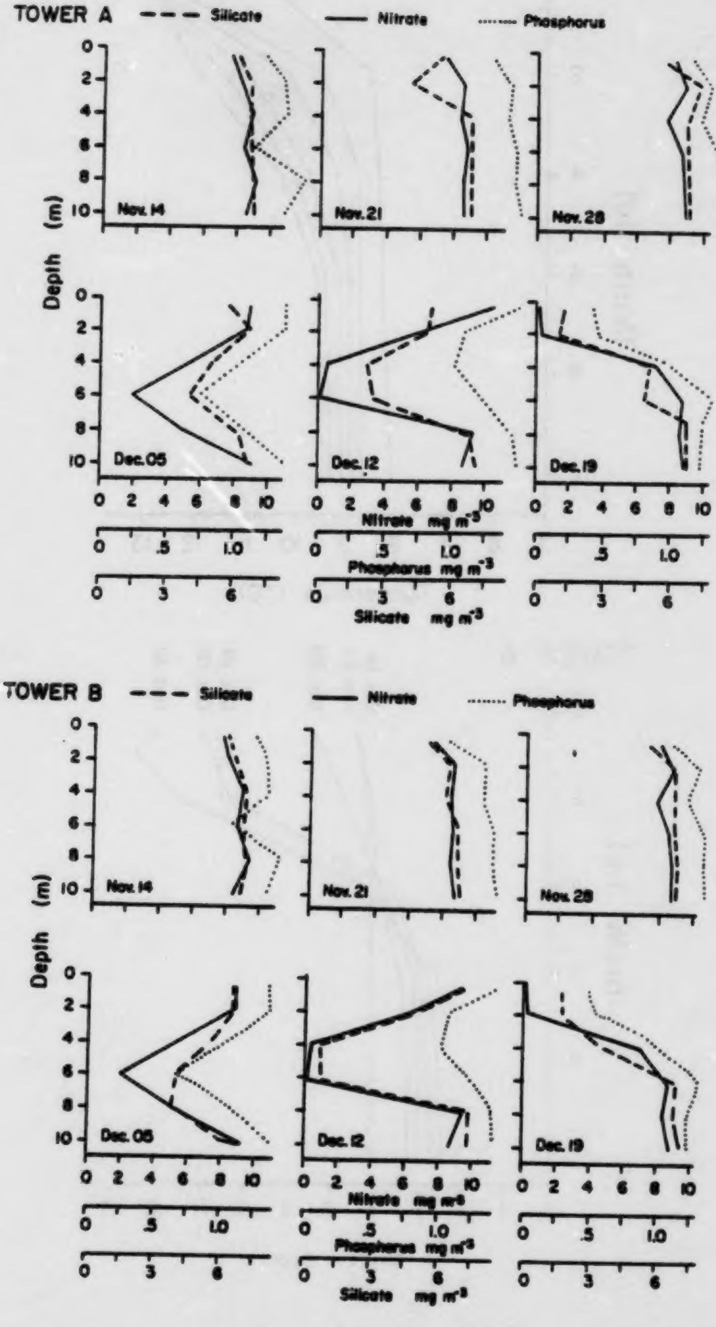

Figure 4. Vertical profiles of nutrient concentrations in Tower A and Tower B from November 14, 1980 to December 19, 1980.

Plankton abundances fluctuated with date in both sides of the tank (Figure 5 and Table 6). Both diatoms and flagellates initially decreased, then diatoms became dominant in Tower A and Tower B. Flagellates declined to almost imperceptible levels in Tower A on November 28 and December 5. At the end of the experiment, in both sides of the tank, diatoms decreased to approximately one-half of their peak abundances and flagellates increased over mid-experiment levels.

Zooplankton in both sides of the tank exhibited peak abundance by developmental stage. By depth, copepodite maximum abundances coincided with maximum peaks of chlorophyl concentration. Nauplii were most abundant near the surface of the water column. Interestingly, the copepodites were not as apparent in Tower A as they were in Tower B. This may indicate that there was heterchrony in their development. In terms of mean abundances over all dates, Pseudocalanus minutus was the dominant adult copepod and nauplii and copepodites, which were not identified to species, were the next most abundant animal groups. Other adult zooplankton species from highest to lowest mean abundance were: Oithona similis, Temora longicornis, Acartia spp. Eurytemora affinis, Centropages spp.-Tortanus discaudatus and Evadne normanni. Generally, the relative abundances of the zooplankton species were similar in both sides of the tank. Macrozooplankton consisted of polychaete larvae which were more numerous in Tower B than in Tower A.

In summary, there was considerable variability with depth and date in both sides of the tank. All variables except macrozooplankton exhibited higher mean levels in Tower A than in Tower B (Table 6). This increase is probably a direct result of the nutrient enrichment. There appeared to be two distinct time periods in the dynamics of both communities. With the exception of marcozooplankton, both plankton communities exhibited similar relative abundance patterns and this similarity did not seem to be swamped by nutrient enrichment effects. In comparing the laboratory and field communities, the tower tank communities possessed substantially fewer species than did the field.

The loop diagram of the tower tank communities is given in Figure 6. There are five differences between it and the field diagram. First, S (Sagitta-Calanus) was not included in the laboratory diagram because these animals did not occur; the tower tank is probably too shallow for the depth distinction found in the field to be important. The self-damping of S was transferred to M (macrozooplankton). Second, a silica (Si) variable has been added because silica was not functioning in a similar way to phosphate in the tower tank. The inclusion of the Si variable does not alter any of the other results, however, its presence allows predictions of the fate of this nutrient in the tower tank. In the field data set, there were no silica data and thus, its inclusion as a separate variable in the field loop diagram, was not motivated by the data or a need to explain particular theoretical results. Third, the interaction between Z_1 and Z_2 is a two-way one in the laboratory diagram. Fourth, Z_3 is not self-damped at least when macrozooplankton are present. The majority of copepod individuals are consuming phytoplankton and detrital foods (18, 21, 22) especially when populations have high relative abundances of juvenile stages. Fifth, the interaction between N_1 and A_2 is also diagrammed as a two-way flow. Biologically, this means that the flagellates are increasing through their consumption of phosphate and that their rate of change in

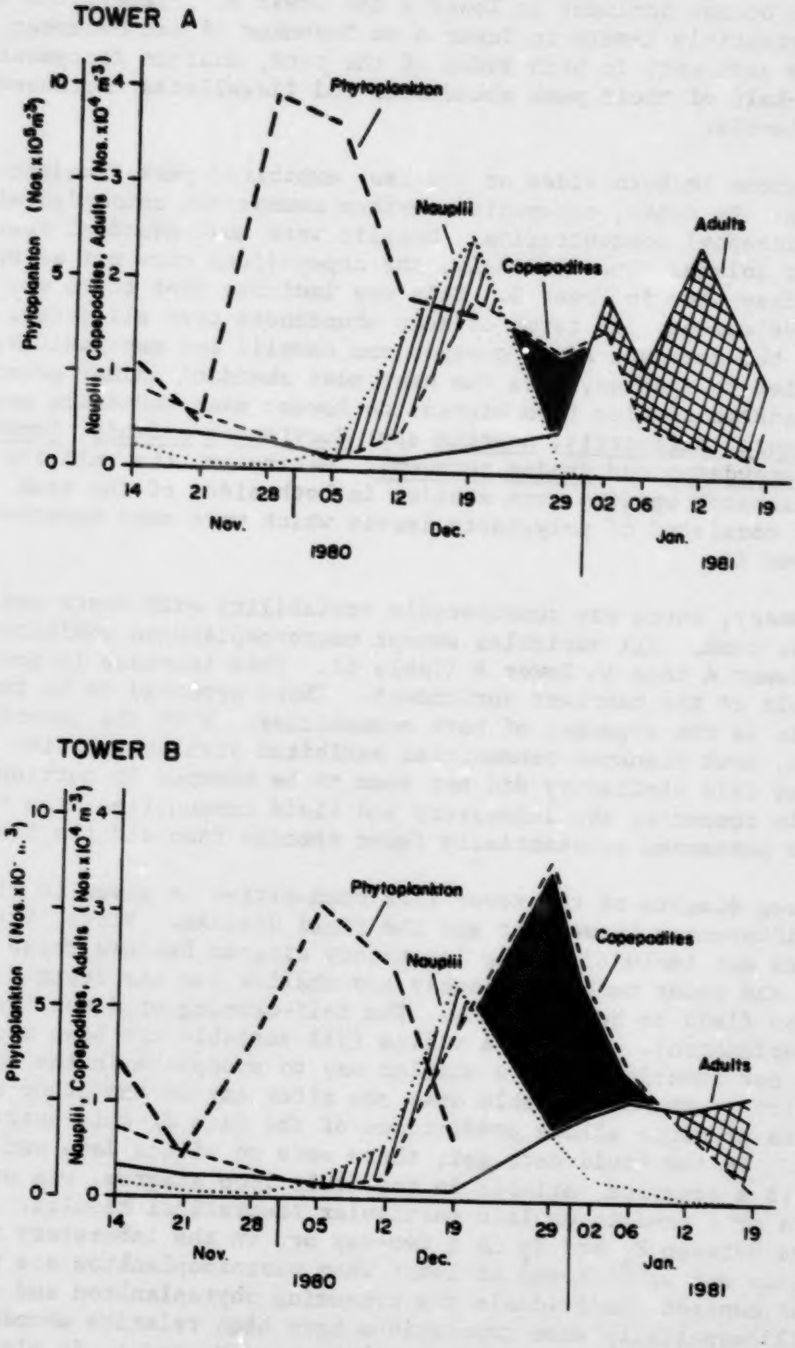

Figure 5. **Changes in phytoplankton and zooplankton abundances in Tower A and Tower B. Phytoplankton were not enumerated after December 19, 1980.**

TABLE 6. ABUNDANCES AND CONCENTRATIONS OF VARIABLES IN THE EXPERIMENTAL (A) AND CONTROL (B) PARTS OF THE TOWER TANK*

Variable ↓	11/14	11/21	11/28	12/5	12/12	12/19	$\bar{X}$	Date →
N_1(A)	1.35	1.13	1.47	1.14	1.01	1.53	1.27	
N_1(B)	1.27	1.33	1.32	1.19	1.28	0.94	1.22	
N_2(A)	9.29	8.03	10.5	6.19	5.91	5.88	7.63	
N_2(B)	8.58	8.48	8.52	6.48	5.82	5.58	7.24	
S_1(A)	6.63	5.95	6.68	5.68	5.20	4.19	5.72	
S_1(B)	6.45	6.38	6.13	5.51	4.28	4.50	5.54	
A_1(A)	768.	183.	2,440.	2,180.	1,310.	1,200.	1,350.	
A_1(B)	715.	96.0	1,410.	2,260.	1,730.	749.	1,160.	
A_2(A)	74.5	9.28	.1	.1	6.19	12.4	17.1	
A_2(B)	24.9	6.19	6.19	6.19	.1	24.8	11.4	
Z(A)	29.2	21.1	17.8	10.6	21.8	50.9	25.2	
Z(B)	23.6	16.4	11.7	9.34	14.0	45.8	20.2	
M(A)	542.	208.	28.0	0.0	0.0	0.0	130.	
M(B)	555.	264.	57.0	28.0	7.0	14.0	154.	

* Key: N_1 (phosphate), N_2 (nitrogen), and S_1 (silica) concentrations are calculated as mg - at $N\text{-}NO_{3/m}3$, mg - at $P\text{-}PO4/m^3$ and mg - at $Si\text{-}SiO_3/m^3$, and A_1 (diatoms), A_2 (flagellates), Z_A (zooplankton) are calculated in 10^3 organisms / m^3, macrozooplankton are given as number /m^3.

relation to this nutrient is more apparent in the laboratory than in the field. It is unknown how changes in species composition and diversity affect the interaction terms. Alternatively, the need to include the N_1 to A_2 pathway may be a result of the change in location of the entry of the parameter input to the community network. In both sides of the tower tank, the major parameter inputs occurred at A_1 (diatoms) for Time 1 as compared to an N_2 entry point in the Bedford Basin diagram. The ecological nature of the parameter input is not known, however, it undoubtedly relates to factors influencing the vertical heterogeneity of A_1. Some realistic, potential factors are light, temperature and turbulence. From the viewing ports of the tower tank, it was possible to observe the stratification directly. At 2m it was not possible to see the divider, whereas at 3m the water was clear and the view unobstructed.

The testing of the loop diagram shown in Figure 6 was accomplished by comparing the directed changes in standing crop values determined experimentally with those predicted by the theoretical analysis (Table 7). Whereas, it was possible to compare predicted and actual correlation values for the field diagram, the laboratory system fluctuated markedly and correlation matrices in the early weeks were not similar to those calculated for the final weeks. As Figure 5 illustrates, there was a great deal of negative correlation between abundance curves of pairs of planktonic components. Thus, the directed change method of testing gave a more effective comparison with a temporal sequence data frame. The theoretical predictions for Time 1 were the same for both sides of the tower tank and were consistent with the known and observed biology. The

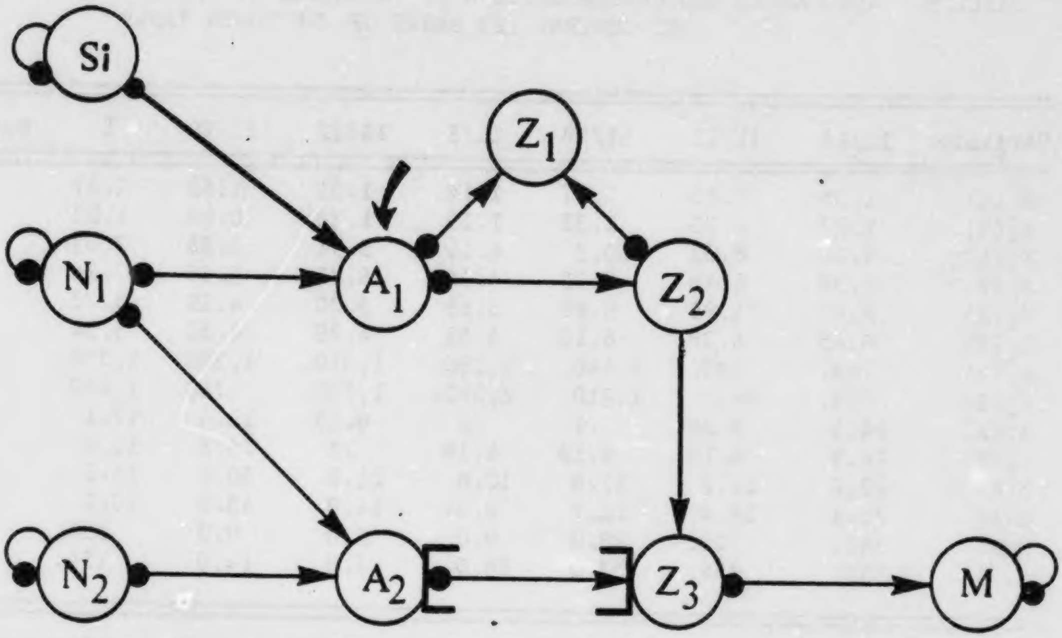

Figure 6. Loop diagram of the plankton community in the tower tank during Time 1. The curved arrow indicates where a positive parameter input enters the network. Predictions for this input are given in the first column of Table 7. All symbols are the same as those given in Figure 2, except that Si (silica) is separated from N_1 (phosphate complex). The brackets around the negative loop of length two between A_2 and Z_3 indicate that this loop may weaken in the laboratory.

TABLE 7. COMPARISON OF DIRECTED CHANGES IN STANDING CROPS OF TOWER TANK VARIABLES (A = EXPERIMENTAL, B = CONTROL) WITH PREDICTIONS FROM LOOP ANALYSIS OF DIAGRAM IN FIGURE 5. TIME 1 INCLUDES DATA FROM 11/14/80 TO 11/28/80, AND TIME 2 INCLUDES DATA FROM 12/12/80 12/19/80

Increase in:	Predicted Change			Data Changes			
▾	Time 1	Time 2		Time 1		Time 2	
	A and B	A	B	A	B	A	B
	$+A_1$	$-Si$	$+A_2$				
N_1	-	+	-	-	-	+	-
N_2	?*	-	-	-	-	-	-
Si	-	-	+	-	-	-	+
A_1	+	-	-	+	+	-	-
A_2	?*	+	+	-	-	+	+
Z_1	+	-	-	**	**	**	**
Z_2	-	+	+	-	-	+	+
Z_3	-	+	+	-	-	+	+
M	-	0	+	0	-	0	+

Key: Symbols are given in the legends to Figure 2 and Table 4.
* If the A_2 - Z_3 loop weakens, then these changes would not be ambiguous; N_2 would increase and A_2 would decrease.
** Z_1 was not measured in these experiments.

changes in N_2 and A_2 levels were ambiguous because there is a positive pathway of effect through Z_2 and Z_3 and a negative pathway through N_1.

In Time 2, the network of Tower A changed because the macrozooplankton were not present (not shown). The self-damping of the macrozooplankton variable was probably transferred to Z_3. The loop between A_2 and Z_3 was stronger than it had been in Time 1. The major parameter input entered through a negative change in Si concentration. The predictions are compared with the actual qualitative data changes in Table 7. If Z was not self-damped then the change in A_2 would be predicted to be 0 instead of the increase that is predicted with Z_3 self-damped and which was also found by the empirical results. Increased self-damping may have arisen because of the proportionally higher abundances of adults in the zooplankton community that occurred at the end of the experiment. The presence of self-damping, however, was not experimentally determined. In the control side (Tower B), the network did not change but the parameter input entered at A_2. The most likely explanation is related to the vertical heterogeneity of the phytoplankton in regard to factors such as light, turbulence, and temperature.

DISCUSSION

Two examples have been presented to demonstrate how a qualitative methodology (loop analysis) can be applied to marine plankton communities in the field and laboratory. The underlying motivation of this study is to develop better ways of understanding complex ecological systems without resorting to a "measure everything" approach which is always costly and often ineffective. Improved understanding and methodology are essential for environmental assessment. The Discussion is centered around some of the advantages and problems with using loop analysis in an ecological context. In some cases, these categories are not discrete entities. Since the purpose of the paper is to illustrate "by example," the discussion is also organized this way rather than presenting a full literature review of the many biological results and conclusions of the study.

ADVANTAGES OF LOOP ANALYSIS

Coping with Complexity

Lawlor (2) estimates that there is less than one change in a googol of constructing an ecosystem with a random-number generator. An aquatic environment can possess a 1000 species; thus in the sense of Lawlor (2) the networks in the water may exceed the sand grains on the beach. The complexity problem is enormous. Lawlor (2) suggests that since the problem cannot be tackled by computer, it is more reasonable to confine exploration and study to those systems that are biologically acceptable. If a large enough group of these systems can be analysed, then basic ecological properties and structural similarities should emerge. Loop analysis has tremendous potential to make complexity manageable at a low cost relative to other methods. Loop diagrams can be constructed and tested with minimum data requirements. The results of loop analysis are more easily-transferred among ecosystems than are those of the more traditional quantitative methodologies. (Computer simulations often work only within a narrow set of parameter values and initial conditions found in a single ecosystem). It will also be possible to use loop analysis to develop a set of ecosystem descriptors for community structure and stability; for example, the average connectivity for a system of N variables or ratios of feedback at each level of N - K variables would be useful.

Understanding Biological Phenomena

Because the qualitative nature of the ecosystem is emphasised in loop analysis, it is easier to focus on the critical components and pathways of effect in understanding ecosystem structure and function in contrast to quantitative methodologies. Many different types of variables can be included in one diagram and there is no need to standardize everything into a single unit such as kilocalories of energy or grams of carbon. For example, abiotic and biotic variables were included in the Bedford Basin diagram. Variables can be also used which cannot be measured. Human actions such as management decisions or institutions such as regulatory agencies can be diagrammed with plankton populations.

In this study, the Bedford Basin loop diagram explained most of the correlation patterns that were observed during an annual cycle. The plankton community appears to be well-organized and receiving a major parameter input through the N_2 variable. In the laboratory, it was possible to study the network further and to delineate important structural differences between the field and laboratory communities. The laboratory experiment provided a detailed analysis of a small section of the annual cycle in comparison to the field data that integrated the annual results. In many regards, the dynamics of the tower tank communities resembled those of field communities undergoing spring bloom conditions (30-32). At the beginning of the bloom there are low temperature and light levels that begin to rise. Turbulence has often been involved as a mechanism to explain nutrient increases in the surface waters. Seasonal succession proceeds with a shift from small to large diatoms to flagellates and from microzooplankton to larger zooplankters. Flagellates appear after nutrient levels become depleted and thermal stratification occurs. In both the experimental and control sides of the tower tank, major parameters enter through the algal populations and not through nutrient variables. Thus, despite the differences in nutrient input and the resultant differences in biomass levels, the qualitative plankton dynamics were similar for both Tower A and Tower B. Events other than nutrient enrichment were driving the networks. This is interesting in contrast to many of the disputes in the literature on the roles of particular factors in the spring bloom. (The shift from the major parameter input entering at nutrient to one entering at the algal level has been found in a qualitative study of eutrophication in Gull Lake (Lane, unpubl.); perhaps this parameter shift represents a fundamental phenomena in aquatic ecosystems).

By integrating the field and laboratory results with the theoretical framework provided by loop analysis, it is possible to understand the complex community dynamics of systems undergoing stress more effectively than by using a single data set. Extrapolation from laboratory results to field situations is often difficult. The laboratory diagram is similar to the field diagram and differences in the variables and linkages were directly related to the measured biological differences. This also confirmed the sensitivity of the methodology.

Guide to Research

Loop analysis also provides a guide to research in that it generates testable hypotheses and elucidates particular variables and interaction links that need further study. A few examples are given from the present study; the list is not inclusive. 1) The tower tank system experienced little turbulence; it has often been given an important role in spring succession dynamics. Is turbulence important? Was the low level of turbulence related to the increased stratification of phytoplankton which in turn led to a shift in the node of entry of the main parameter input? Conversely, does the parameter input at algae occur regardless of the levels of turbulence and/or prevailing nutrient ratios in the field? These questions could be answered with more experiments and theoretical analysis. 2) What are the major mechanisms underlying parameter input to algal variables? 3) Do subtle shifts in nutrient ratios change either the structure or the node of parameter entry in these networks? 4) How much self-damping is occurring; for example, how many animal populations exhibit cannibalism within their functional group? 5) Under what circumstances is A_2-Z_3 a strong loop and when can it be disregarded? 6) What is the role of biomass level in the qualitative dynamics that were observed in both sides of the tank? When do enhanced levels of biomass lead to structural changes in the network? 7) In this experiment, when M disappeared in Tower A, there were few changes in the predictions. The loss of other variables, however, could have more dramatic consequences on the network and loop analysis predictions. When will the loss of a variable be important?

Applying loop analysis to these field and laboratory systems provides a wealth of interesting ideas for future research and study. Often analysis of loop diagram yields important predictions about particular links in the network or delineates an omitted variable. Lane and Levins (3) demonstrate how in a 4 variable-9 link system only four links need to be measured to understand the critical pathway of effect. Data sets are not used to correct equations or achieve better fitting parameters but rather to find new ways to look at systems and to find new variables of interest. In subsequent research, there can be more selection in collecting the missing information and the "measure everything" approach does not need to be invoked.

Ecological Causality and Prediction

Loop analysis helps greatly in understanding cause and effect in ecological systems. "Causality, however, is always a theoretical construct: as Hume observed, causation cannot be proved empirically. Whatever the observed evidence, there remains the possibility that some uncontrolled factor was responsible for the occurrence" (33). Because loop analysis gives direct predictions in regard to the standing crops, turnover rates (not shown, (3) and their correlation patterns, it is useful for a variety of assessment and management problems. Loop diagrams provide needed focal points, and causal pathways can be drawn out and examined. Loop analysis also provides an integrative tool for predicting the effects of multiple indirect pathways between pairs of variables that are not directly related to each other.

PROBLEMS WITH LOOP ANALYSIS

Integration of Theory and Biology

Whereas, loop analysis permits an effective integration of theory and biology, the results reported in this study should also serve as a warning. The subtle shifts in parameter inputs over small time scales and the qualitative changes in interaction signs can occur even when the species list and relative abundance patterns are fairly consistent. A great deal of biology is needed to elucidate these subtle effects. Loop analysis assumes that variables with similar time dynamics are grouped together in the networks. This assumption needs substantially more testing than it has received to date. For example, from both freshwater and marine results, it appears that most fish populations are acting as parameters, not variables, in regard to the plankton populations. It may be that fish are sufficiently slower so as to be "disconnected" from the plankton dynamics. Whereas, loop analysis can help delineate where biological detail needs to be supplied, it is not a magic antedote that will solve all of our environmental problems. Good biological insight is needed as much as it has ever been.

Testing Loop Analysis Results

This study used the most simple, direct form of comparing theoretical and empirical results possible-inspection. Statistical testing is presently receiving considerable effort in our research group and will be reported in more detail later. At present, it is not possible to state if the best diagram for the observed results has been constructed. 8% of the field results were in error by a complete sign change. It is not known 1) what the universe of possible networks is like, 2) when a particular level of agreement is satisfactory, and 3) how to separate errors of prediction from sampling errors. There is also much work needed in delineating the attributes of particular types of data sets and how they can be interrelated in regard to testing loop predictions. For this study the two data sets were fairly consistent in regard to the networks that were generated. This may not always occur.

ACKNOWLEDGMENTS

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Comments on
Using Qualitative Analysis to Understand Perturbation to Marine Ecosystems in the Field and Laboratory

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The work summarized in this contribution by Lane complements that discussed in the previous paper by Puccia and Bennett. Both studies involve the application of loop analysis methodologies to the study of marine ecosystems, and both are components of a single, overall research program funded by the Environmental Protection Agency. Because of the interrelatedness of the two studies, many of my comments in response to the previous paper apply to the present one also. My comments here will focus primarily on additional insights into the ecological utilization of loop analysis brought out by this paper. As before, work described here is in progress and not complete. My comments should again be interpreted in light of this fact.

Whereas the previous paper focused on the structure of marine benthic communities, Lane is concerned here with planktonic sytems in a small, semi-enclosed marine basin located offshore from Nova Scotia. Also, in contrast to the largely theoretical nature of the previous paper, Lane demonstrates how theoretical loop analyses can be combined with both field and laboratory data into an overall research program on plankton dynamics. Correlations derived from field data on abundances of planktonic organisms and essential elements are used to test predictions of state variable correlation patterns generated by loop analyses of a network model of the plankton community. Similarly, data resulting from laboratory manipulations of plankton community dynamics using experimental "towers" are used to test loop model predictions of changes in standing crops of select state variables following altered parameter inputs. Thus, Lane provides examples of ways in which loop model predictions can be tested, and shows how the loop model approach can provide direction to field research programs, thereby avoiding the pitfalls of what she correctly terms the "measure everything" philosophy which has characterized much ecosystem research in the past (especially that which has had simulation modeling as a major goal or component). In this way, Lane demonstrates how loop analysis can provide an essential link between theory and experimentation in ecology. Another contrast between this paper and the previous one relates to the structure of the models studied: the models used here are structurally more elaborate, and include essential elements and several categories of autotrophs as state variables in addition to strictly invertebrate compartments.

As with the previous paper, this contribution raises several questions concerning the ecological utilization of loop analysis which should be addressed in subsequent work. First, the question of the isolatability of a portion of the ecosystem for study via loop analysis again requires consideration. Although the network models used here are more "complete" than those used in the previous paper, they still ignore key components of ecosystems (e.g., pathways and state variables involved in the breakdown of detritus and the regeneration of essential elements). Such variables again enter the present analysis only indirectly as "parameter inputs." Yet inclusion of such variables in the network structure could grossly change model predictions. For example, inclusion of pathways for detritus production and remineralization would add indirect, positive pathways from algae to essential elements, and might thereby alter model predictions. Again, it would seem as though all major ecosystem components required for a given analysis should be included explicitly in the loop models to allow meaningful predictions to be made. Conceptually treating portions of the ecosystem as being separable or isolatable from the remainder of the network strucutre is a questionable procedure, and may lead to erroneous predictions.

A second problem in this use of loop analysis again involves what might be considered "extra" or ad hoc hypotheses. In her paper, Lane stresses the need to determine the major entry point(s) for human interventions into ecosystems—i.e., the state variables though which the altered parameter input(s) is(are) "entering" the system. Yet little discussion is provided as to how this is done in a rigorous or objective fashion. How, for example, does one determine that the major parameter input to the "field" loop model is through variable N_2, or through variable A_1 to the "laboratory" loop model? On what objective basis does one determine that the major parameter input for "Time 2" has changed, or that it differs for the two towers? Also, does the approach taken preclude multiple "parameter inputs" to the system of interest? Perhaps due to the brevity of the discussion, the approach taken here appears tentative and arbitrary. Again, this problem could be resolved by including all ecosystem components relevant to a given analysis explicitly as state variables rather than implicitly (and arbitrarily) as parameter inputs.

A third problem raised by this paper concerns the model dependence of loop analysis results. Lane correctly emphasizes that, as is the case in all modeling studies, results of her loop analyses are dependent on the structure of the models employed. Even small changes in model structure could possibly lead to substantial changes in model predictions. However, in contrast to the previous paper, this work does not include an analysis of alternative model structures, so that one does not know how sensitive (or robust) these results are. Additionally, different loop diagrams for the field and laboratory planktonic assemblages are presented, without thorough discussion of how the model structures were constructed from the available data in each case. Also the author suggests that the connection between variables A_2 and Z_3 may "weaken" in the laboratory assemblages, without explaining how this was determined. All of these questions suggest that additional attention must be given to objective means for constructing loop diagrams, and to investigating the sensitivity of model predictions to network structure.

The use of correlation analyses to verify or text the prediction of loop analyses is another area requiring additional study and clarification. The validation of models, whether qualitative or quantitative, is a non-trivial problem which has generally received inadequate attention by systems ecologists. Although the fact that a large number of correlations in the field data set were correctly predicted by the associated loop model may be reassuring, it is not necessarily a rigorous test of model validity. Because of the large number of direct and indirect pathways involved in a network model of any degree of structural complexity, an "incorrect" model structure could "correctly" predict a number of inter-variable correlations. Thus, the whole issue of validating loop models requires further study. As suggested in my response to the Puccia-Bennett paper, methods for validating such qualitative models will probably be quite different from methods employed to validate quantitative simulation models.

Finally, some comments concerning the issue of ecosystem complexity as raised by Lane must be made. It should be pointed out that complexity is a property of a scientific description of a given system; it is not in any sense "inherent" in the system. Models of natural phenomena which are broadly recognized as being useful or successful are such because they focus on essential details of a given phenomenon, and ignore other related but "nonessential" information. Ecosystem models (in the broadest sense) continue to appear complex because ecologists persist in describing ecosystems in terms of species-level properties and interactions, and not as a functionally intact system with indentifiable macroscopic properties. The number of species or interaction pathways says nothing about ecosystem complexity. Nor is the improbability of randomally constructing a "reasonable" biological network structure via computer experiment a major challenge to ecosystem theory. Such approaches ignore two essential sources of constraint on current ecological networks: constraints imposed on contemporary biotic assemblages by historically antecedent structures, and constraints imposed on biotic evolution by system-level matter and energy exchanges with the surrounding physicochemical environment. Biotic structures of ecosystems evolve over long time periods from prior structures under the constraints of biogeochemical interactions between the biota as a whole and the physicochemical environment to which that biotic structure is intimately coupled. They do not emerge de novo via any sort of random arrangement of members of a local species pool. It is a worthy goal to seek "non-complex" descriptions of ecosystems. However, current ecosystem models are complex because ecologists have generally failed to achieve an acceptable conceptual simplification of these systems and not because ecosystems are inherently more complex than other systems studied by science.

To summarize, Lane has provided a stimulating illustration of the coupling of theoretical loop models with field and laboratory experimental data in a study of marine plankton dynamics. Her work nicely complements that of Puccia and Bennett, and demonstrates again that loop analysis is an effective research tool for elucidating ecosystem properties. The approach also has real potential for impact analysis of ecosystems, but a potential which largely remains to be demonstrated conclusively.

NEW APPROACHES TO MEASURING THE BIOLOGICAL IMPACT OF ENVIRONMENTAL TOXICANTS

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OVERVIEW AND GENERAL REMARKS

Current methods for assessing the impact of polluting chemicals and environmental contaminants are limited in scope and generally are not well suited to predicting biological impact under real life situations, since the exposure is generally occurring simultaneously with a variety of other types of stress (nutritional, chemical, pathogenic). The work to be described is part of a collaborative study to construct an integrated numerical predictive model of wild small mammals outdoors from components recently developed by the group. The model can be used to study the complex interactions between physiological stress due to climate, chemical toxicity, and pathogens. Predictions of the effects of climate, toxicity and pathogens on growth, reproductive potential and activity periods as well as food and water needed for survival, growth and reproduction can be generated from the model and tested experimentally. The theoretical-experimental model should be useful ultimately as a quick screening device and field monitor. The model incorporates a state-of-the-art breakthrough in predicting heat and mass transfer of animals with furry insulation in almost any outdoor environment. It also includes a microclimate model, a predictive outdoor respiratory water loss model, a physiological thermoregulation model and both fractional and full factorial experimental designs that provide rapid, effective, simultaneous screening of many variables whose effects must be empirically determined. Most of the first six months were spent getting needed preliminary data in the laboratories of the four participating colleagues so as to launch our first series of integrated, multifactoral experiments.

Although the division of labor in making the presentation may give the impression that each group is working independently, we can assure you that we are integrating our efforts and findings to a high degree in order to complete our original objectives. The work will be discussed in three parts as shown in your program and we will attempt to provide sufficient background information to make clear why certain approaches, techniques and agents have been chosen over others in developing the model.

Comments on
New Approaches to Measuring the Biological Impact of Environmental Toxicants

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The objective of this proposal is to develop a model system which would predict the effect of a combination of naturally occurring environmental insults on the growth and reproductive success of a small mammal under laboratory and field conditions.

The three variables in the study were to be climate, pathogens and toxic substances. In order to carry out a study with such varied disciplines, it was necessary to recruit the expertise of several different investigators. Dr. Porter was to carry out the heat loss studies and climatic stress difference, Dr. Yuill had responsibility for pathogens and their effect on the host's general health and reproductive capacity. Dr. Hinsdill was to assess the effects of toxic chemicals on the same parameters. All of the data and experimental design were to be examined by Dr. Hunter, who has a wide range of experience in statistics.

Dr. Porter's group was to establish certain baseline data on deer-mice in metabolic chambers. These data were to include heat production, O_2 consumption, CO_2 production and evaporative water loss under various climatic conditions, disease states and toxicant exposures. Once these data were collected, long-term experiments in the Biotron (controlled environmental chamber) could be carried out to determine the ultimate effect of the various combinations of insults on the growth and reproductive success of the deer-mice.

Dr. Yuill's group was responsible for determining the effective non-lethal levels and routes of administration (infection) for acute and chronic virus and bacterial infections. They were then to collaborate with Dr. Hinsdill's group to determine appropriate combinations of pathogen and toxicant that would allow the animals to survive for the duration of the study period thus allowing the assessment of these sublethal infections and intoxications on the long-term health and reproductive success of deer-mice. The pathogens chosen for these studies included viruses which produced both acute and chronic infections and bacteria which produce acute infections. By taking blood samples at various intervals throughout the experiment it would be possible to monitor the production of antibody and interferon in the animals under different conditions of environmental stress.

The third phase of the study was to be carried out by Dr. Hinsdill and consisted of determining immunosupressive non-lethal dose levels of potential chemical toxicants. Two compounds (cyclophosphamide and azathioprine) were selected as positive controls due to their known immunosupressive action. The unknown chemicals chosen belong to a group of chemicals which are relatively unknown in terms of their effects on mammalian systems. These are the plant growth regulators Maleic hydrazide, cycloheximide,and triiodobenzoic acid.

IMPACT OF TOXICANTS, DISEASE AND CLIMATE ON GROWTH AND REPRODUCTION USING *PEROMYSCUS MANICULATUS*

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INTRODUCTION

Many biotic and abiotic variables that affect animal's capacity to grow and reproduce have been identified. How to efficiently study and model the effects of such a large number of variables in a biological system is a difficult problem. We have found that an energy and mass budget framework provides an overall context in which we can examine and model many diverse factors affecting the capacity of animals to grow and reproduce.

One cornerstone to this approach is the development of mechanistic models of heat and mass exchange between an organism and its environment. These exchanges and the energy and mass balances which result are of prime importance to survival, growth, and reproduction. By modeling these exchanges we can understand the limits they impose upon many aspects of an organism's life.

These exchanges take place by fairly simple physical mechanisms and can be described by mechanisic models (1). These models describe heat and mass exchange as a function of the microclimate and certain relevant physical and physiological features of the animal. They are then used to drive other models of interest, such as food and water requirements, activity patterns, body temperature, and dessication rate. This approach has been used to study temperature and water regulation in ectotherms and the constraints those regulations impose upon an ectotherm's use of time and space (1,2,3,4,5,6), and species distributional limits (7).

A second cornerstone to our approach is the use of highly efficient full and fractional factorial designs to explore biological variables that can not be defined with heat and mass balance equations. We have completed experiments using those fractional factorial designs, to test model predictions of climatic effects and to identify the significant "black box" variables affecting the ability of female deer mice, *Peromyscus maniculatus* , to raise young to weaning (8,9,10). All significant variables were then used in full factorial designs to establish a response surface. These designs are ideally suited for screening large numbers of variables simultaneously and then iteratively adding resolution when the data suggest that additional information is needed.

We are now integrating these designs with our heat and mass transfer models to predict effects of toxicants and disease on maintenance, growth, and reproduction requirements for animals in any physical environment. To do this we are working in three major areas: 1. Heat and water loss for small mammals 2. Food and water assimilation rates for small mammals 3. Growth and reproduction studies of small mammals. Heat and mass transfer equations have been used to predict maintenance food and water requirements of healthy adults of several species (1,2,3), but prediction of climatic effects of growth and reproduction potential has only been done for healthy invertebrate ectotherms (10,11) and fish (12).

MODELS OF GROWTH AND REPRODUCTION

Our model of animal growth and reproduction is derived from the conservation of energy and mass for animals. Simply stated, the energy available for growth and reproduction is the difference between the energy assimilated from food and that which is metabolized to carbon dioxide, water and heat. Figure 1 (Porter and McClure, in preparation) is a system of word equations representing the mass and energy balances for an animal. It shows the linkage between the physical environment, physiological processes and their biological consequences. We expect toxicants and diseases to affect several variables described below.

HEAT BALANCE EQUATION

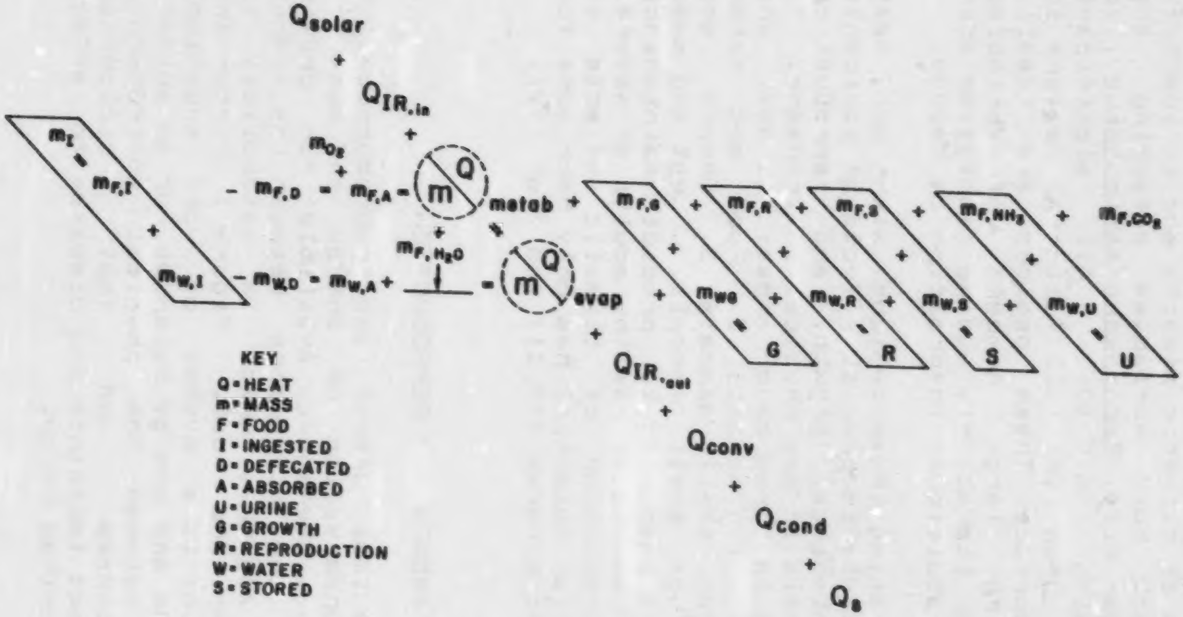

Q solar
+
Q IR,in
+
m O2
+
m I = m F,I + m W,I
− m F,D = m F,A =
Q / m metab
+
m F,G + m F,R + m F,S + m F,NH3 + m F,CO2
− m W,D = m W,A +
m F,H2O
= Q / m evap
+
m WG = G
m W,R = R
m W,S = S
m W,U = U
Q IR,out
+
Q conv
+
Q cond
+
Q S
KEY
Q = HEAT
m = MASS
F = FOOD
I = INGESTED
D = DEFECATED
A = ABSORBED
U = URINE
G = GROWTH
R = REPRODUCTION
W = WATER
S = STORED

Figure 1. Coupled heat and mass balance equations (Porter and McClure, 1981).

The equation on the diagonal is the heat transfer equation for an animal. Once each term on the diagonal has been evaluated using the physics of heat and mass transfer (13,14,15). The equation can be solved for body temperature or metabolic rate. The variables, Qmetab and Qevap, are the heat generation and loss terms due to physiological processes. They are also influenced by environmental heat inputs and losses. Examples of environmental heat inputs are solar and infrared radiation, and losses, Qsolar and QIR,in. An example of heat loss is infrared emission from the animal, QIR,out. Heat exchange also occurs by the mechanisms of convection and conduction, Qconv and Qcond. The storage of heat, Qs, may be either positive, negative or zero, depending on whether body temperature is rising, falling, or at steady state. The physiological variables Qmetab and Qevap each have a flow of mass associated with them. The top horizontal equation is the flow of mass due to the dry component of food. The bottom horizontal equation is the mass flow of water.

MASS BALANCE EQUATIONS

The mass of food ingested less that defecated is the amount of mass absorbed (mF,I - mF,D = mF,A). Absorbed mass that is oxidized (symbolized by the slash) produces heat (Qmetab), metabolic water, (mF,H2O), protein decomposition products (mNH2+), and carbon dioxide, (mF,CO2). The remaining mass may be used for activity, growth, reproduction or stored in the form of fat. The metabolic rate requires a second mass flow other than food, namely oxygen, which accepts hydrogen ions at the end of the cell mitochondrial respiratory chain to synthesize water (mF, H2O).

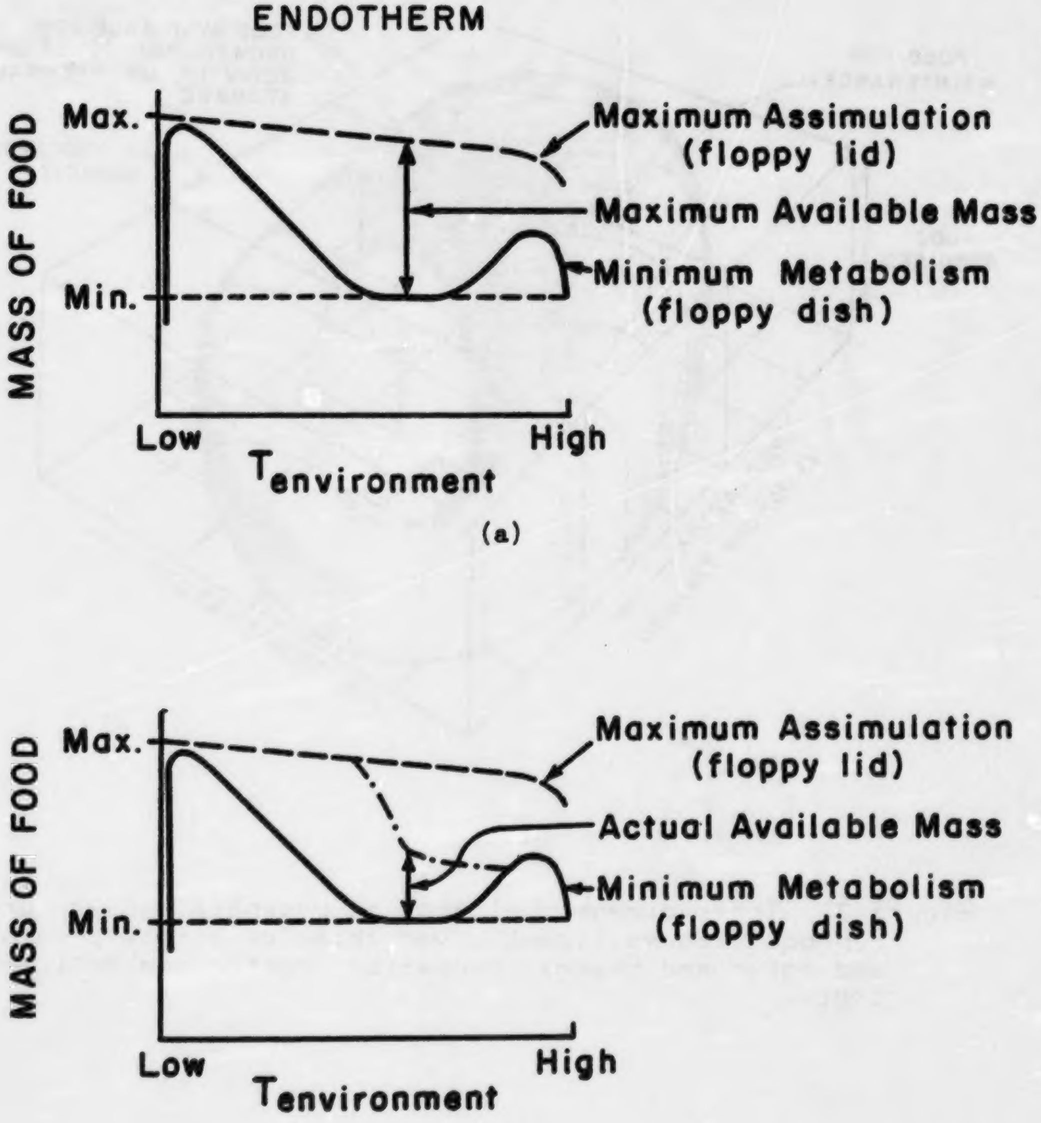
The mass of water ingested less that lost in defecation equals water absorbed (mW,I - mW,D = mW,A). Total water available, however, is the sum of absorbed water plus metabolic water from food. Thus the mass flow equations are not only coupled to the heat transfer equation by physiological processes, but are also coupled to each other at the following six locations: food intake, metabolic water production, growth, reproduction, fat storage and urine production. In this coupled system of equations there are many fewer degrees of freedom than if the equations were all independent. Thus, a change in one variable effects changes throughout the system. Thus, experiments must be designed to take this interdependence into account. By modeling the total resources assimilated (left hand side in Figure 1) and the obligatory heat and mass losses (the center terms in Figure 1) we can predict the maximum resources left over for growth, reproduction or storage. Our strategy is to first estimate the limits imposed by climatic effects on heat and mass exchanges and then study how the limits are modified by non-climatic factors (e.g., toxicants, disease, social factors, food quality, litter size, and activity level).

GRAPHICAL REPRESENTATION OF EQUATIONS

The minimum cost surface (resting metabolic rate) of endotherms is often plotted as a function of air temperature. The minimum metabolic rate is determined by the interaction between an animal and physical climatic variables (e.g., air temperature, wind speed, solar radiation). For example, to remain homeothermic (Figure 2a), an endotherm must increase its resting metabolic rate as the gradient between itself and its thermal environment grows large (e.g., exposure to cold or hot air temperatures).

The upper line, maximum assimilation, must be empirically determined. The space between the lower resting metabolism line and the upper maximum assimilation line is the maximum available mass for use in growth, reproduction or fat storage as described by (16,17). Actual available food assimilated may be reduced by ecological factors (Figure 2b) resulting in less available mass. The reduction illustrated here could be due to reduced prey availability at high temperatures. Toxicants or disease could induce similar reductions via effects on the senses, locomotion ability, digestion or absorption processes. When these calculations are done relative to different combinations of air temperature and radiation, rather than the black body conditions illustrated in Figures 2a and b, three dimensional surfaces appear, as shown in Figure 3.

Figure 3 illustrates the complex effects of climate on the mass balance of an endotherm. The lower bowl-shaped surface defines the food required for maintenance for all combinations of air temperatures and radiant loads for a given wind speed. The maintenance surface, the "floppy dish", is a dynamic surface that rotates clockwise in the horizontal plane as animal size increases or wind speed decreases and rotates counterclockwise with smaller animal size or as wind speed



(a)

(b)

Figure 2. Two dimensional plot of maintenance and available food mass vs temperature.

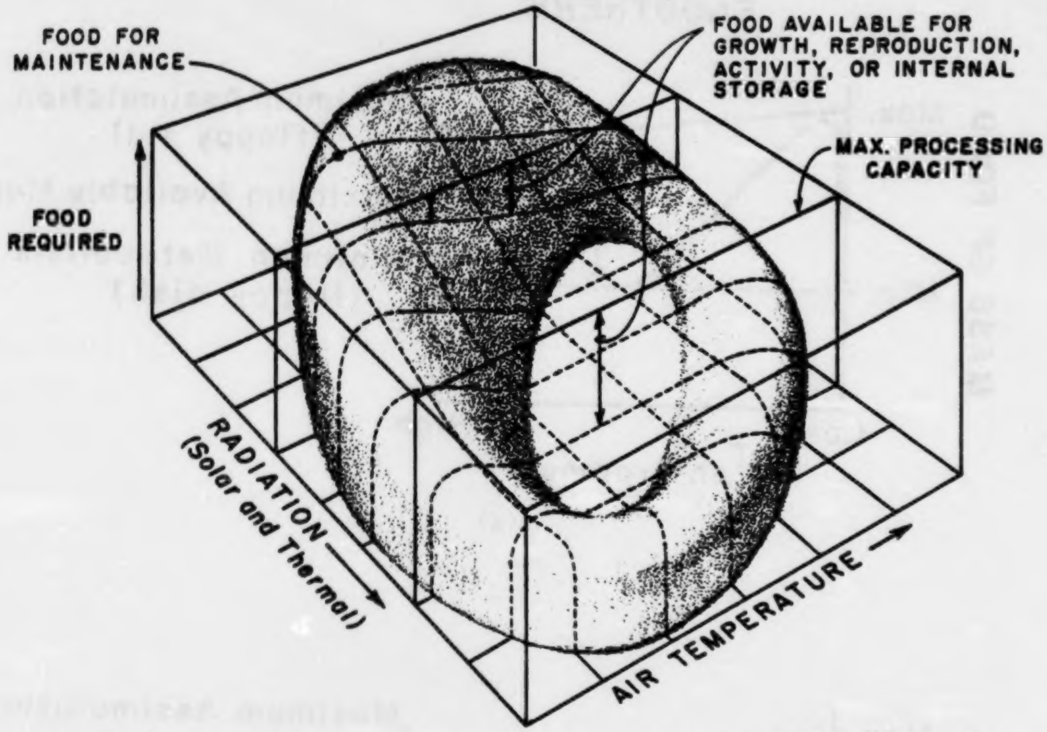

FOOD FOR MAINTENANCE
FOOD AVAILABLE FOR GROWTH, REPRODUCTION, ACTIVITY, OR INTERNAL STORAGE
MAX. PROCESSING CAPACITY
FOOD REQUIRED
RADIATION (Solar and Thermal)
AIR TEMPERATURE

Figure 3. Three dimensional plot of potential growth or reproduction vs climatic variables of air temperature and solar and thermal radiation (Porter and McClure, 1981).

increases. The maintenance surface can be visualized starting with the solid diagonal line from the "cold" corner to the "hot" corner. The diagonal is the minimum metabolism curve of Figure 2, where air temperature equals radiant temperature. This is the standard environment found in metabolic chambers. When it is cold, metabolism and food required is maximal (cold corner). As environmental temperatures increase, metabolism decreases, until the "flat" thermoneutral zone is reached. As temperatures continue to increase, the thermoneutral zone is traversed, and metabolism begins to increase with heat stress as the "hot" corner is approached. The remaining dish surface is the food required when air and radiant temperature are not equal. The edges of the surface that drop off represent lethal conditions. New coupled mechanism fur models developed by our group at Wisconsin were instrumental in generating surfaces like this (18,19,20,21).

The upper surface forming the top of Figure 3 represents the maximum food processing capacity of the animal given unlimited food. This physiological lid is represented as being flat, but drops somewhat at high temperatures for deer mice, Figure 4. Actual consumption may not reach the lid unless the animal is a lactating female (22, 23), is exceptionally active, is severely cold stressed (17), or laying down fat for hibernation.

The space between the two surfaces, the availability space, represents the "uncommited" food mass which can be used for growth, reproduction, storage or activity. The rules governing the trade-offs between growth, reproduction, storage and activity are still largely unknown.

We have developed models of the maintenance surface for fur-covered endotherms. These models have three substantial improvements over the models used to generate the climate space of (24). First, the convective component for the floppy dish was adjusted for Reynolds number range (a combined velocity, body size effect) and the convection coefficient was adjusted for turbulence outdoors, which may affect heat transfer by 50% or more (25,26). Second, the fur heat transfer model is radically different. The fur model for the climate space was only a conduction model based on still air conductivity. We now know that radiation losses through the fur can account for 50% or more of the heat loss (27). Also radiative and conductive heat losses through fur are coupled in complex

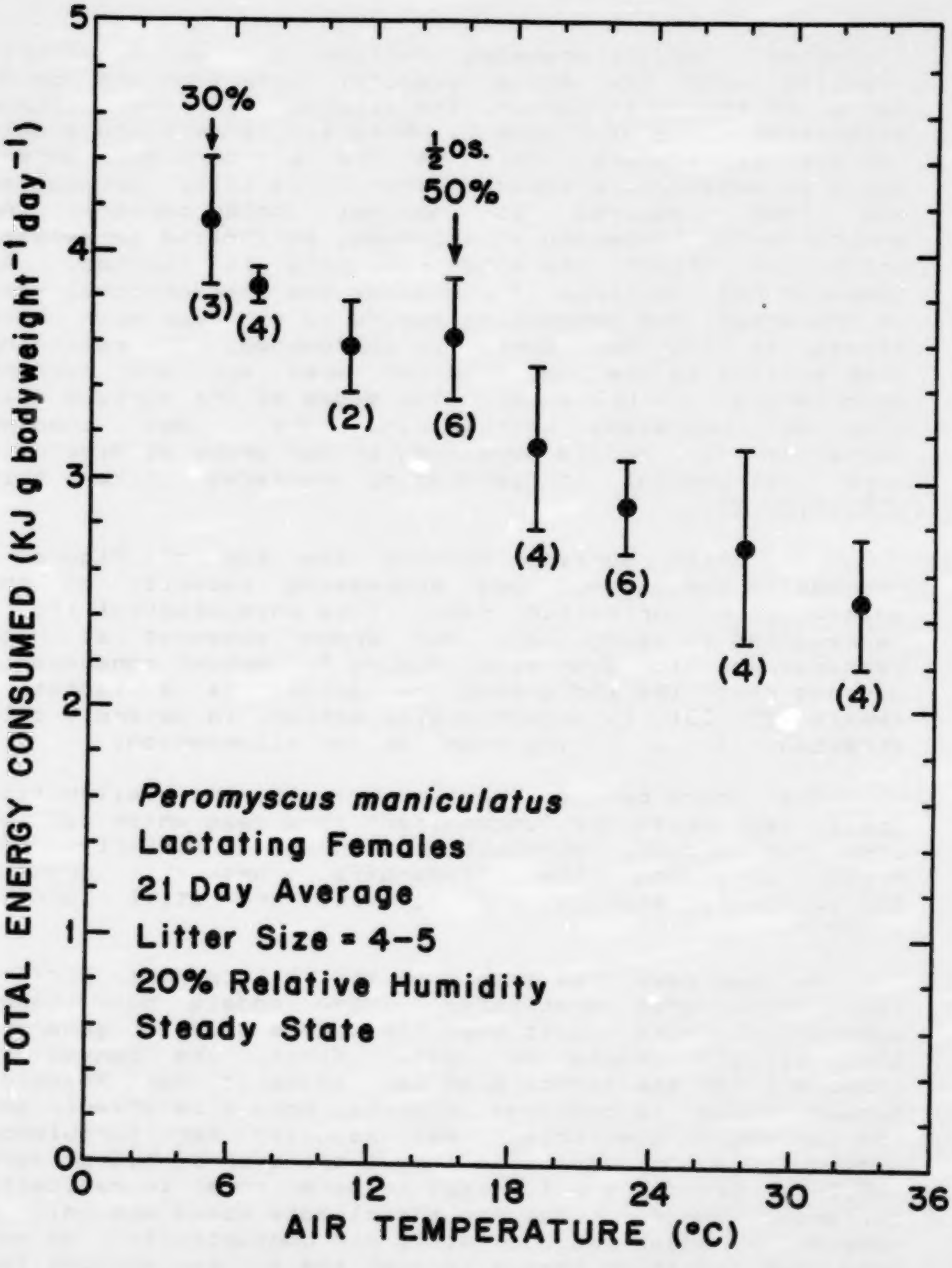


Figure 4. Maximum ad lib energy consumption during lactation vs air temperature. Radiant temperature equals air temperature.

wave that we have recently have been able to model (19,20,21). Third, we have developed a model that relates body temperature, limb blood flow and posture to skin temperature. Using these improvements, we can now predict metabolic rates of deer mice to within +/- 6% of measured values in metabolic chambers (Figure 5a, Conley and Porter, in preparation). Individual variation in data points can now be identified as due to variation in body temperature and limb blood flow. Figure 5b illustrates postural effects on heat loss at different temperatures (Conley and Pórter, in preparation).

EXPERIMENTAL METHODS

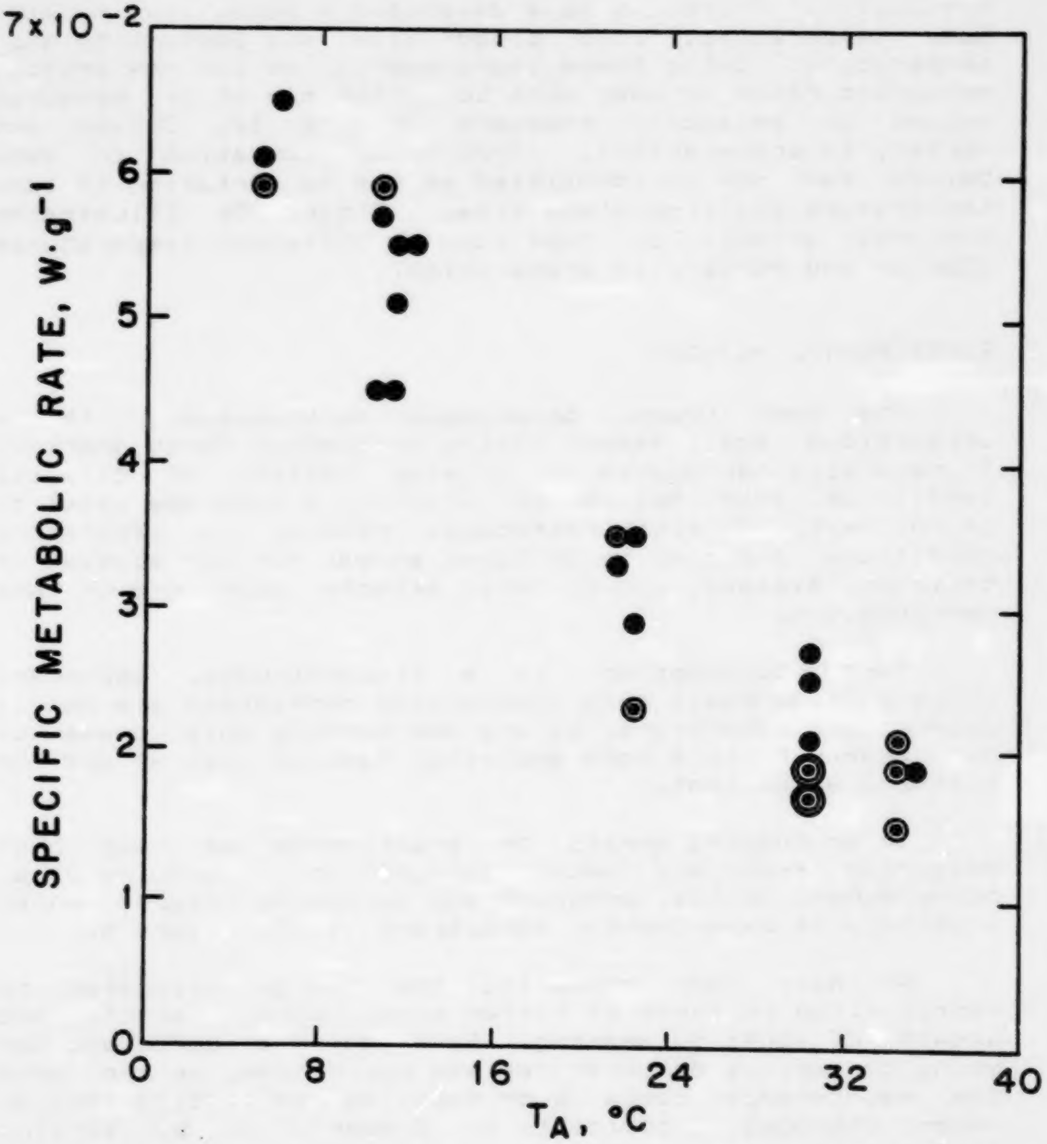
The deer mouse, Peromyscus maniculatus, is a ubiquitious small mammal living throughout North America. It naturally reproduces in a wide variety of climatic conditions from Mexico to Alaska and from sea level to 14,000 feet. It also reproduces readily in laboratory conditions and thus is an ideal animal for our studies of toxicant, disease, and climatic effects upon growth and reproduction.

Food consumption is a discontinuous, voluntary process whose basic rate controlling mechanisms are poorly understood. Therefore, we are approaching this phase of our research in a more empirical fashion than we did for heat and water loss.

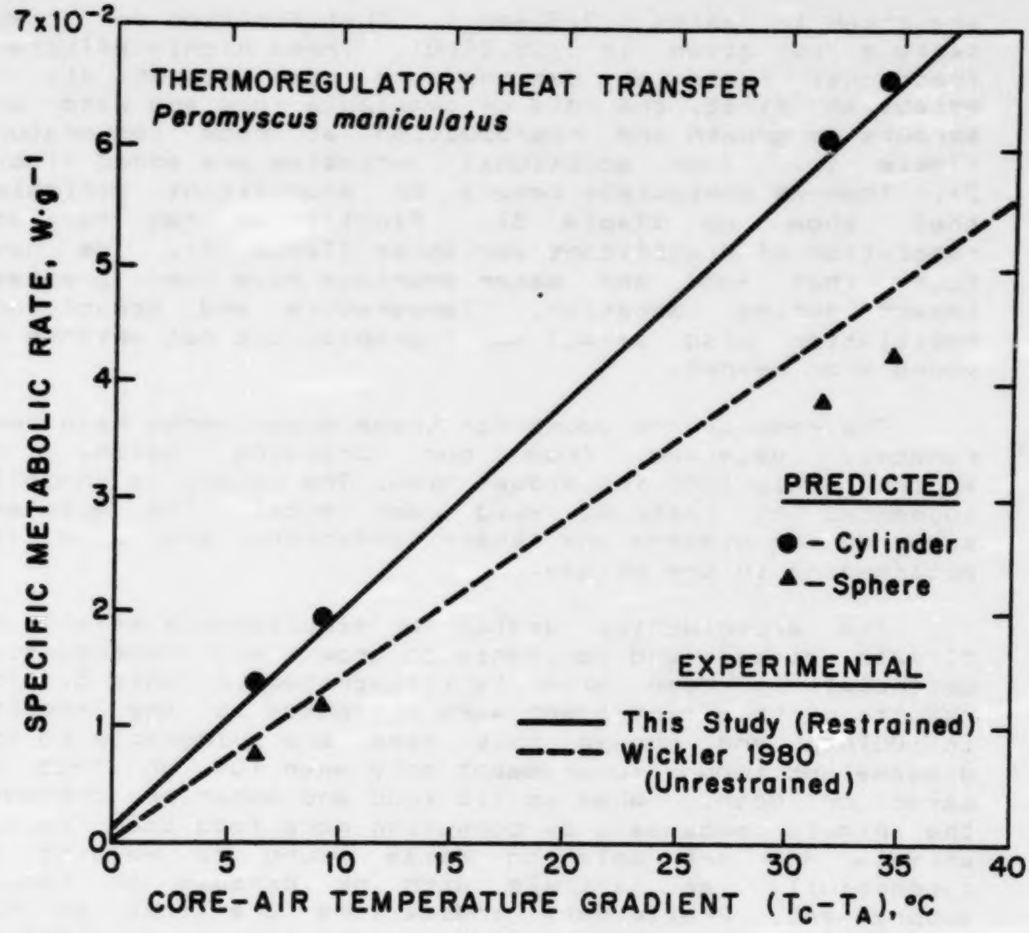
In an ongoing series of experiments we have been measuring food and water consumption of growing mice, non-pregnant adults, pregnant and lactating females under a variety of experimental conditions (8,9), Figure 4.

We have been measuring the energy allocated to reproduction in terms of litter size, birth weight, and growth of young to weaning. By exposing a female and her young to various temperatures and humidities, we can move the maintenance costs up or down. By restricting food or water, introducing toxicants or disease, or by varying food quality (i.e., presence or absence of sprouts) we can raise and lower the assimilation level. This gives us a direct measure of how climate, toxicants, disease, food, and water interact to affect growth and reproduction (Figure 2).

Designs we have used for studies of growth and reproductive capacity of pregnant and lactating females



(a)



(b)

Figure 5. Comparisons of observed and predicted metabolic rates for different limb blood flow rates and postures vs air temperature (Conley and Porter, in preparation).

are shown in Tables 1,2,3,and 4. Justification for these designs is given in (28,29,8). These highly efficient fractional factorial designs are used iteratively to establish first, the role of available food and water and sprouts on growth and reproduction at room temperature (Table 1). Then additional variables are added (Table 2). Then we manipulate levels of significant variables that show up (Table 3). Finally we get complete resolution of significant variables (Table 4). We have found that food and water shortage have their greatest impact during lactation. Temperature and temperature oscillation also affect survivorship, but not weights of young when weaned.

The females and young for these experiments have been randomly selected from our breeding colony of approximately 1000 individual mice. The colony is annually augmented by captured wild deer mice. The mice are screened for disease and their offspring are used for outbreeding in the colony.

The experimental design for simultaneous effects of climate, disease and toxicants on growth and reproduction potential in deer mice is illustrated in Table 5. The results of this experiment were presented at the meeting in Duluth and showed that mice are vulnerable to the disease and immune suppressant only when low on food or water or both. When ad lib food and water are present, the animals compensate by consuming more food than healthy animals and are able to raise young to weaning as successfully as animals with no disease or immune suppressant. Preliminary indications are that serious effects of this disease and toxicant show up at times of food or water shortage and are most severe when both food and water are in short supply. Thus screening tests done on non-reproducing animals fed ad lib food and water may likely give misleading predictions that the agents are harmless.

TABLE 1. INITIAL DESIGN FOR ASSESSING THE EFFECTS OF FOOD AND WATER ON DEERMOUSE GROWTH AND REPRODUCTIVE SUCCESS

	Variable	(-) Levels	(+)	Center Replicate
1	Amount of food	80%	Ad lib	90%
2	Type of dry food	Regular	Breeder	1/2 & 1/2
3	Amount of water	60%	Ad lib	80%
4	Sprouts	None	Ad lib	50%

	Variable			
Run #	1	2	3	4
1	-	-	-	-
2	+	-	-	+
3	-	+	-	+
4	+	+	-	-
5	-	-	+	+
6	+	-	+	-
7	-	+	+	-
8	+	+	+	+

TABLE 2. DESIGN FOR PRECOPULATION TO WEANING EXPERIMENT IN THE BIOTRON

(*Peromyscus maniculatus*)

	Variable	(−) Levels	(+)	Center Replicate
1	Temperature time dependence	Steady state	Oscillating	1/2 Osc
2	Temperature (C)	11(+/-5)	23(+/-5)	17.5 +/- 2.5
3	Relative Humidity	90%	20%	50%
4	Number of young left with mother	2	4	3
5	Nest box	No	Yes	No roof
6	Amount of food	80%	Ad lib	90%
7	Amount of wheat sprouts	None	Ad lib	50%
8	Amount of water	80%	Ad lib	90%

	Variable							
Run #	1	2	3	4	123* + 5	124* + 6	134* + 7	234* + 8
1	−	−	−	−	−	−	−	−
2	+	−	−	−	+	+	+	−
3	−	+	−	−	+	+	−	+
4	+	+	−	−	−	−	+	+
5	−	−	+	−	+	−	+	+
6	+	−	+	−	−	+	−	+
7	−	+	+	−	−	+	+	−
8	+	+	+	−	+	−	−	−
9	−	−	−	+	−	+	+	+
10	+	−	−	+	+	−	−	+
11	−	+	−	+	+	−	+	−
12	+	+	−	+	−	+	−	−
13	−	−	+	+	+	+	−	−
14	+	−	+	+	−	−	+	−
15	−	+	+	+	−	−	−	+
16	+	+	+	+	+	+	+	+

*Specific three factor interaction confounded with the main effect of the variable

TABLE 3. SECOND ITERATION OF DESIGN IN TABLE 2

	Variable	(-) Levels (+)		Center Replicate
1	Temperature time dependence	Steady state	Oscillating	1/2 Osc
2	Temperature (C)	11(+/-5)	23(+/-5)	17.5 +/- 2.5
3	Relative Humidity	90%	20%	50%
4	Number of young left with mother	2	4	3
5	Nest box	No	Yes	No roof
6	Amount of food	80%	Ad lib	90%
7	Amount of wheat sprouts	None	Ad lib	50%
8	Amount of water*	60%	80%	70%

Variable

Run #	1	2	3	4	123 + 5	124 + 6	134 + 7	234 + 8
1	-	-	-	-	-	-	-	-
2	+	-	-	-	+	+	+	-
3	-	+	-	-	+	+	-	+
4	+	+	-	-	-	-	+	+
5	-	-	+	-	+	-	+	+
6	+	-	+	-	-	+	-	+
7	-	+	+	-	-	+	+	-
8	+	+	+	-	+	-	-	-
9	-	-	-	+	-	+	+	+
10	+	-	-	+	+	-	-	+
11	-	+	-	+	+	-	+	-
12	+	+	-	+	-	+	-	-
13	-	-	+	+	+	+	-	-
14	+	-	+	+	-	-	+	-
15	-	+	+	+	-	-	-	+
16	+	+	+	+	+	+	+	+

The blocking pattern is the same as in previous runs.

*The levels of this variable are the only ones different from Table 2

TABLE 4. THIRD ITERATION OF DESIGN IN TABLE 2

	Variable	(-) Levels (+)		Center Replicate
1	Temperature time dependence	Steady state	Oscillating	1/2 Osc
2	Temperature (C)	11(+/-5)	23(+/-5)	17.5 +/- 2,5
3	Relative Humidity	90%	20%	50%
4	Number of young left with mother	2	4	3
5	Nest box	No	Yes	No roof
6	Amount of food	80%	Ad lib	90%
7	Amount of wheat sprouts	None	Ad lib	50%
8	Amount of water	80%	Ad lib	90%

	Variable							
Run #	1	2	3	4	123 + 5	124 + 6*	134 + 7	234 + 8
1	-	-	-	-	-	+	-	-
2	+	-	-	-	+	-	+	-
3	-	+	-	-	+	-	-	+
4	+	+	-	-	-	+	+	+
5	-	-	+	-	+	+	+	+
6	+	-	+	-	-	-	-	+
7	-	+	+	-	-	-	+	-
8	+	+	+	-	+	+	-	-
9	-	-	-	+	-	-	+	+
10	+	-	-	+	+	+	-	+
11	-	+	-	+	+	+	+	-
12	+	+	-	+	-	-	-	-
13	-	-	+	+	+	-	-	-
14	+	-	+	+	-	+	+	-
15	-	+	+	+	-	+	-	+
16	+	+	+	+	+	-	+	+

* Signs for the level of food administered have been reversed from Table 2 for full resolution of all possible interactions with food (Box, Hunter and Hunter, 1979).

TABLE 5. FIRST EXPERIMENTAL DESIGN TO ASSESS MAIN EFFECTS AND INTERACTIONS OF FOOD, WATER, SPROUTS, AN IMMUNE SUPPRESSANT AND A DISEASE

	Variable	(-) Levels (+)		Center Replicate
1	Amount of food	80%	Ad lib	90%
2	Amount of water	80%	Ad lib	90%
3	Amount of sprouts	None	Ad lib	50%
4	Immune suppressant *	No	20 mg/kg	10 mg/kg
5	Virus **	No	ID 100	ID 50

	Variable				
Run #	1	2	3	4	1234 + 5
1	-	-	-	-	+
2	+	-	-	-	-
3	-	+	-	-	-
4	+	+	-	-	+
5	-	-	+	-	-
6	+	-	+	-	+
7	-	+	+	-	+
8	+	+	+	-	-
9	-	-	-	+	-
10	+	-	-	+	+
11	-	+	-	+	+
12	+	+	-	+	-
13	-	-	+	+	+
14	+	-	+	+	-
15	-	+	+	+	-
16	+	+	+	+	+

* Cyclophosphamid

** Venezuelan equine encephalitis (vaccine strain)

ID100 means infectious dose yielding 100% infected animals

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Comments on
Impacts of Toxicants, Disease, and Climate on Growth and Reproduction Using *Peromyscus maniculatus*

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The objectives of this research include the following:

1) To develop a quantitative model to assess impacts of toxicants, pathogens and climate on small mammal growth and reproduction in the Laboratory and in the field.

2) To identify physiological parameters that can be used a) to screen toxicants in the laboratory and field, b) to assess mechanisms of interaction where synergistic effects are found.

These objectives are particularly relevant to EPA objectives since the goal of the research is not only to understand an animal's response to the stress of toxicants and disease but to predict the response. There is a critical need to make realistic predictions concerning the effect of a host of potential toxicants on a variety of animals. Standard toxicity tests are not adequate. For one reason, the laboratory results are not easily translatable to natural populations because of the complexity of biotic and abiotic stresses that field populations are exposed to. Thus, the capability of predicting synergistic effects will be of particular value.

Theoretically, the model proposed is applicable to any endothermic (and with modification, ecothermic) animal. However, the choice of the deer mouse is judicious: 1) the deer mouse is ubiquitous and abundant, 2) it can prosper in highly disturbed habitats such as mine spoils and croplands, and 3) thus it is exposed to a variety of chemical and physical insults. There are probably field populations living under every type of condition imposed by our chemical society. The deer mouse may be viewed as the field version of the white rat. This mechanistic model based upon heat and mass transfer equations (if validated) should be able to predict population fitness of natural populations of deer mice exposed to a variety of contaminants in combination with natural stresses imposed by disease—and over a wide range of altitude and latitude. The idea is exciting! The approach is innovative! And the goals, if achieved, will contribute both to our understanding of species distribution and abundance and to how these parameters are affected by both natural and man-created stresses.

Finally, this research exemplifies good, solid science. There is a theoretical and quantitative model. The methodology is both statistically sound and efficient, using full and fractional factorial designs, and provides rigorous testing. If the model is validated, the theoretical framework is broad enough to permit prediction to be made under a wide range of circumstances. It is good science with a high utilitarian potential.

IMMUNOTOXICITY IN PEROMYSCUS MANICULATUS

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ABSTRACT

Both Swiss-Webster outbred white mice and Peromyscus maniculatus were exposed to a number of plant growth regulators and known immunosuppressive drugs at different levels by two routes. A number of immunological parameters were monitored, including plaque-forming cell counts in the spleen, lymphocyte counts, hematocrit, total white blood cell counts, antibody titers and weights of various organs as well as total body weight. The doses used (mg/kg/day) were as follows: gibberellic acid (GA), 25, 50, 100; chlorocholine Cl (CCC), 1.0, 2.5, 5.0; triiodobenzoic acid (TIBA), 30, 45, 60; maleic hydrazide (MH), 35, 70, 140; Dinoseb (DNBP), 0.4, 0.7, 1.4; and cycloheximide (CH), 3.5, 7.0, 14.0. Cyclophosphamide (CP, 10 mg/kg/day) and azathioprine (AZ, 10 mg/kg/day) served as known immunosuppressant controls. CH, CCC and TIBA most strongly and/or consistently immunosuppressed the mice. CH lowered PFC levels by 60%, reduced hemolysin titers by nearly 50% and lowered thymus weights. CCC and TIBA had less affect but decreased up to 6 different parameters; however, only CH approached AZ or CP suppression levels. DNBP and MH had mixed results. GA, conversely, caused moderate overall enhancement in white mice. On the basis of these results certain compounds and exposure levels were suggested as appropriate for further studies both in Dr. Yuill's laboratory and in the integrated studies supervised by Dr. Porter.

INTRODUCTION

SELECTION OF AGENTS FOR STUDY

A large number of agents were considered for use in developing and testing the model described by Dr. Porter, e.g. heavy metals, pesticides, dioxins, etc. After considering all aspects, including safety for workers, the decision was made to study a selected group of chemicals known as plant growth regulators and to include as known positive-type controls two immunosuppressive drugs which are effective orally and parentally. Plant growth regulators currently account for only a small share of the total worldwide agrochemical market (5-10%). However, a number of experts and business organizations feel that these growth regulators are still in their "infancy", compared to the pesticide market, and that within a few years production and usage will outstrip that of the herbicides. For practical purposes, plant growth regulators can be defined as organic chemicals, other than nutrients, that are applied directly to a plant to alter its life processes or structure in some beneficial way, i.e. to enhance yields, improve quality or, particularly important, to facilitate harvesting. Certain herbicides, when applied in small amounts to induce a specific beneficial change can also be considered plant growth regulators. Some specific examples of usage of plant growth regulators include flowering agent, color enhancer, dormancy breaker, abscission stimulant, fruit-drop controller, fruit-thinner, ripener, and increaser of pod set in soybeans. No one at present can predict accurately to what extent these substances will ultimately contaminate food or our aquatic environment.

At present there are roughly thirty different plant growth regulators being marketed by various chemical manufacturers. Certain of the regulators are produced by more than one chemical company and marketed under different trade names, e.g. maleic hydrazide (MH-30, Uniroyal; Sprout Stop, Sucker Stuff, Drexel; De-Cut, Fairmount). Approximately thirty chemical manufacturers in the U.S. are producing these growth regulators and include such large firms as Dupont, Chevron, Uniroyal, Monsanto, Aldrich, Dow and American Cyanimid. With the continuing need to increase efficiency and decrease costs in agriculture, it is inconceivable that these products will not be heavily promoted. Unfortunately, a literature search reveals that only very limited information is available on the potential toxicity of these compounds for humans or animals. To the best of our knowledge few have ever been examined for their immunotoxicity, nor have they been tested for interactions by the methods proposed here. Table 1 lists six plant growth regulators selected for initial study.

IMMUNOTOXICITY

Toxic agents may obviously affect a variety of organs and tissues, and depending on how such agents are metabolized, affect different species to varying degrees. Almost any agent, however, which would significantly suppress the host defense mechanism, including the immunologically specific cells, could have serious consequences in the animal's ability to deal with foreign material, particularly pathogens. Although chemicals in the environment will continue to be screened for their mutagenic, carcinogenic and teratogenic effects, many investigators are now arguing that these same substances should be screened for immunotoxicity as well. There the consensus ends, however, for there is no simple test or battery of tests which can be relied upon to show the exact nature and degree of immunotoxicity. Part of the reason is the complexity of the immune system as illustrated in Figure 1. Not only are there a great many cell types involved, but there are a number of stages in the immune response which could be affected (Figure 2). Even when it is clearly established that a given compound influences a particular immunological parameter, one is rarely able to predict how this will affect the body's ability to withstand challenge or carry on reproduction. Our model is one of the few that considers this most important aspect of immunotoxicity.

TABLE 1. PLANT GROWTH REGULATORS AND CONTROLS TESTED

	Uses	Crop Applications	Toxicity (Oral)
Azathioprine:	immunosuppressant, chemotherapy	none	
Chlorocholine Cl:	ripener, flowering agent, color enhancer, lodging reducer	sugarcane, pineapple, apples, cabbage, wheat, barley, potatoes, grapes, oranges	LD_{50} = .5-1.0g/kg (mouse)
Cyclohexamide:	abscission agent	most citrus, cotton	LD_{50} = 133mg/kg (mouse)
Cyclophosphamide:	immunosuppressant, chemotherapy	none	
Dinoseb:	herbicide, corn yield enhancer	corn, many crops as herbicide	LD_{50} = 58mg/kg (rat)
Gibberellic Acid:	flowering initiator, shoot stimulator, fruit enlarger, dormancy breaker	grapes, cherries, peas, lemons, wheat, rye, lettuce, potatoes, oats, oranges, beans	LD_{50} >>25g/kg (rat)
Maleic Hydrazide:	growth retardant, sprout inhibitor, lodging reducer	potatoes, tobacco, rye, onions, sugarcane, citrus	LD_{50} = 1400mg/kg (rat)
Triiodobenzoic Acid:	stem thickener, flower induction	soybeans, apples	LD_{50} = 813mg/kg (mouse)

The first step in measuring immunotoxicity or in identifying agents which may have a great biological impact via the immune system is to obtain sufficient information to create an immunological profile similar to that established for cyclophosphamide and shown in Figure 3. Here we see that not only is the production of IgM and IgG immunoglobulin suppressed, but that IgE production has been enhanced. The latter type of antibody is involved in "immediate type" anaphylactic (allergic) reactions and also constitutes a serious threat to the animal. Measuring only immunoglobulin levels may not be sufficient, however, as some animals with depressed levels of lymphocytes may ultimately give normal antibody levels. For this reason, we always include a measurement of the plaque-forming cell ability of the spleen in order to assess whether there are a normal number of cells able to respond to an antigenic stimulus. The assay procedure is summarized in Figure 4.

RESULTS OBTAINED TO DATE

Because of the initial shortage of Peromyscus and because of the fairly large amount of preliminary data needed to begin the multifactoral experiments, outbred Swiss-Webster mice were used for the early experiments. Table

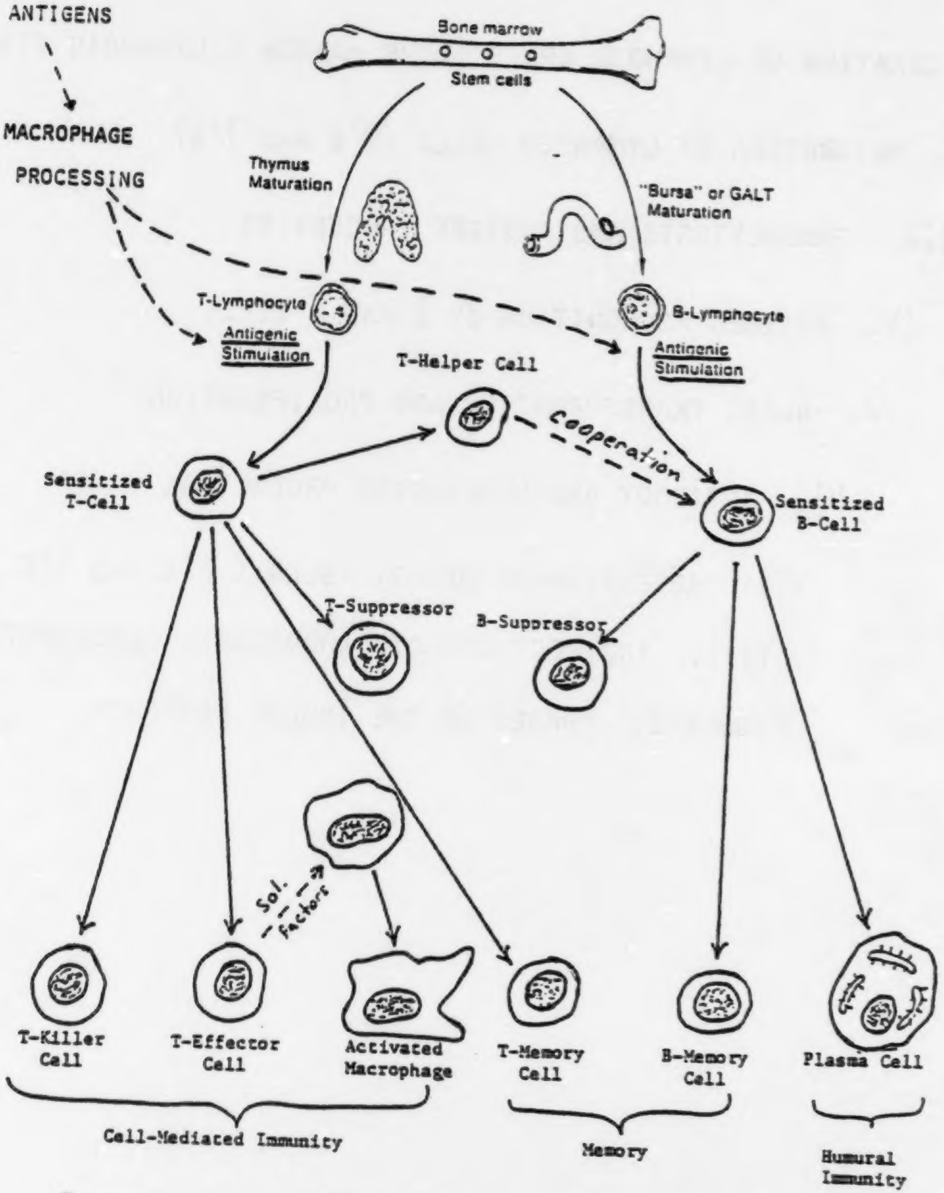
ANTIGENS
MACROPHAGE
PROCESSING
Bone marrow
Stem cells
Thymus Maturation
"Bursa" or GALT Maturation
T-Lymphocyte
Antigenic Stimulation
B-Lymphocyte
Antigenic Stimulation
T-Helper Cell
cooperation
Sensitized T-Cell
Sensitized B-Cell
T-Suppressor
B-Suppressor
Sol. Factors
T-Killer Cell
T-Effector Cell
Activated Macrophage
T-Memory Cell
B-Memory Cell
Plasma Cell
Cell-Mediated Immunity
Memory
Humural Immunity

FIGURE 1. TYPES OF CELLS INVOLVED IN THE IMMUNE RESPONSE.

I. FORMATION OF LYMPHOID CELLS (BONE MARROW & LYMPHOID TISSUES)

II. MATURATION OF LYMPHOID CELLS (B'S AND T'S)

III. PHAGOCYTOSIS AND ANTIGEN PROCESSING

IV. ANTIGEN RECOGNITION BY B AND T CELLS

V. BLAST TRANSFORMATION AND PROLIFERATION

VI. ANTIBODY AND LYMPHOKINE PRODUCTION

VII. RETENTION OF MEMORY CELLS (B'S AND T'S)

VIII. INDIRECT EFFECTS (HORMONES, GLUCOCORTICOIDS)

FIGURE 2. PHASES OF THE IMMUNE RESPONSE.

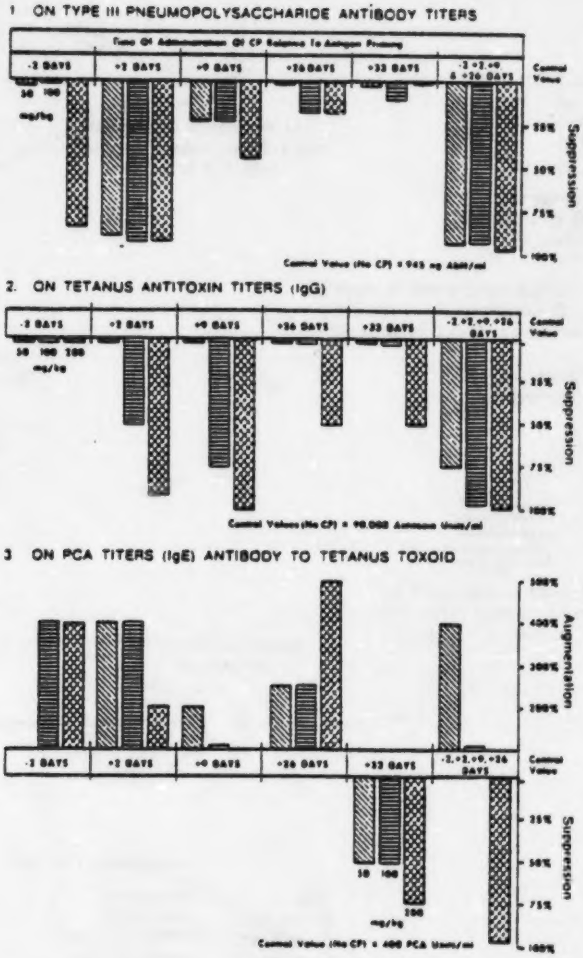

Figure 3. Effects of various doses of intraperitoneally injected cyclophosphamide on several classes of antibodies. Such data constitute an important part of an "immune profile". From R. S. Speirs, D. W. Roberts, R. D. Hinsdill and E. E. Speirs, 1978, Immunotoxicity Assessment, Proc. 4th FDA Science Symposium on Inadvertent Modification of the Immune Response.

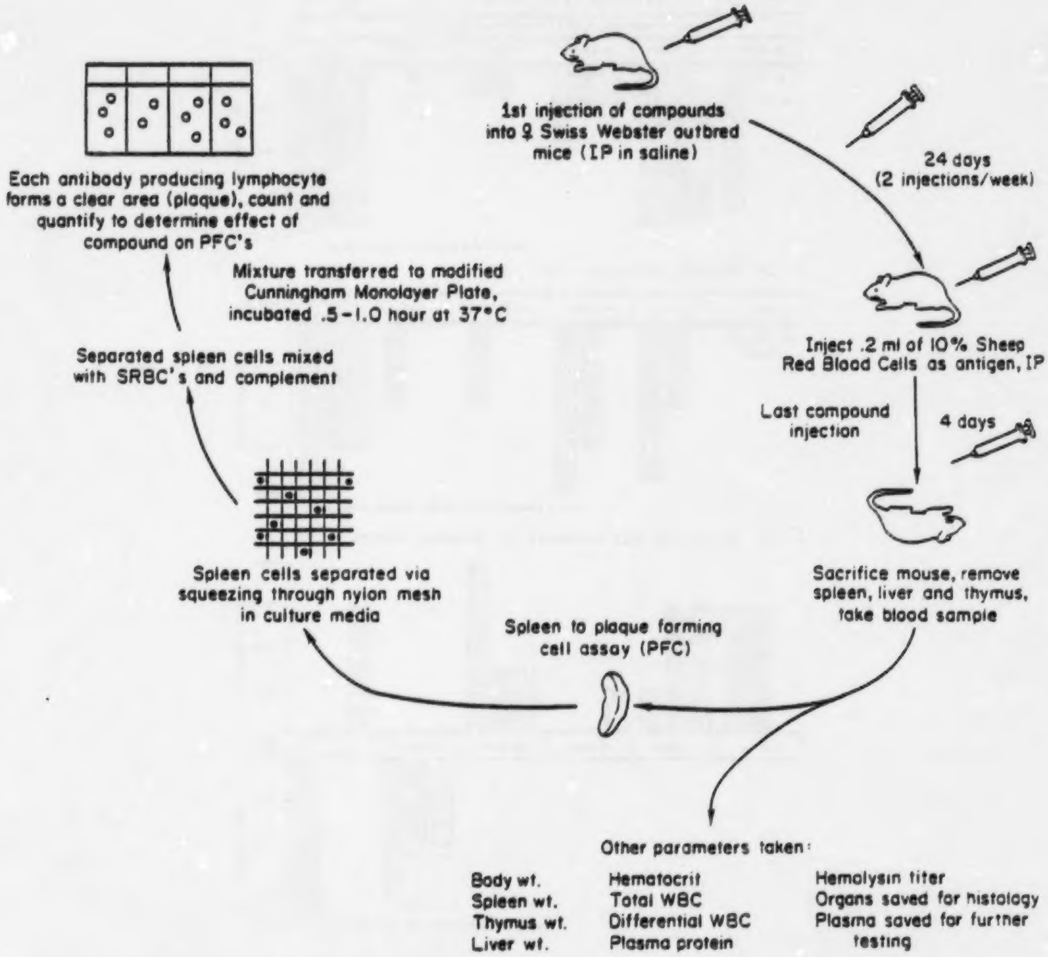
1st injection of compounds into ♀ Swiss Webster outbred mice (IP in saline)
24 days (2 injections/week)
Inject .2 ml of 10% Sheep Red Blood Cells as antigen, IP
Last compound injection
4 days
Sacrifice mouse, remove spleen, liver and thymus, take blood sample
Spleen to plaque forming cell assay (PFC)
Spleen cells separated via squeezing through nylon mesh in culture media
Separated spleen cells mixed with SRBC's and complement
Mixture transferred to modified Cunningham Monolayer Plate, incubated .5-1.0 hour at 37°C
Each antibody producing lymphocyte forms a clear area (plaque), count and quantify to determine effect of compound on PFC's
Other parameters taken:
Body wt.
Spleen wt.
Thymus wt.
Liver wt.
Hematocrit
Total WBC
Differential WBC
Plasma protein
Hemolysin titer
Organs saved for histology
Plasma saved for further testing

FIGURE 4. SEQUENCE OF EVENTS IN THE PGR IMMUNOTOXICITY ASSAY.

2 lists the six plant growth regulators and two known immunosuppressive drugs (acting as positive-type controls) which were injected twice weekly intraperitoneally (IP) for four weeks. To monitor the immunological functions, sheep red blood cells (SRBC) were administered IP on day 24 and the immunological assays were performed on day 28.

TABLE 2. AGENTS AND DOSAGES USED

Agent	Dosage	
Cyclophosphamide (CP)	10	mg/kg/day*
Azathioprine (AZ)	10	mg/kg/day
Gibberellic acid (GA)	25, 50, 100	mg/kg/day
Chlorocholine chloride (CCC)	1.0, 2.5, 5.0	mg/kg/day
Dinoseb (DNBP)	0.4, 0.7, 1.4	mg/kg/day
Cycloheximide (CH)	3.5, 7.0, 14.0	mg/kg/day
Maleic hydrazide (MH)	35, 70, 140	mg/kg/day
Triiodobenzoic acid (TIBA)	30, 45, 60	mg/kg/day

*Injected IP twice weekly for four weeks

Figure 5 shows the effect on the number of PFC's per gram of spleen as a percent of the control value. Note that azathioprine (AZ), gibberellic acid (GA) and maleic hydrazide (MH) appear to stimulate this particular function. This cannot be interpreted as either good or bad. Such findings often indicate that such agents are capable of acting as immunomodulators and that the specific effect at any given time will depend mostly on the dose, but also on the timing of immunization relative to exposure and testing.

Figure 6 shows the effects of the same chemicals on total white blood cell counts, Figure 7 the hematocrit values and Figure 8 the mean lymphocyte counts per gram of spleen. Table 3 is an attempt to interpret and summarize the immunological effects of these chemicals in the usual test setting.

Figure 9 compares feed studies with several agents in both Swiss-Webster outbred mice and Peromyscus. On the basis of these experiments, CP at a feed level of 20 mg/kg/day was recommended as the first toxicant for the integrated studies in both Dr. Yuill's and Dr. Porter's laboratory. 10 mg/kg/day will be used for the center replicate.

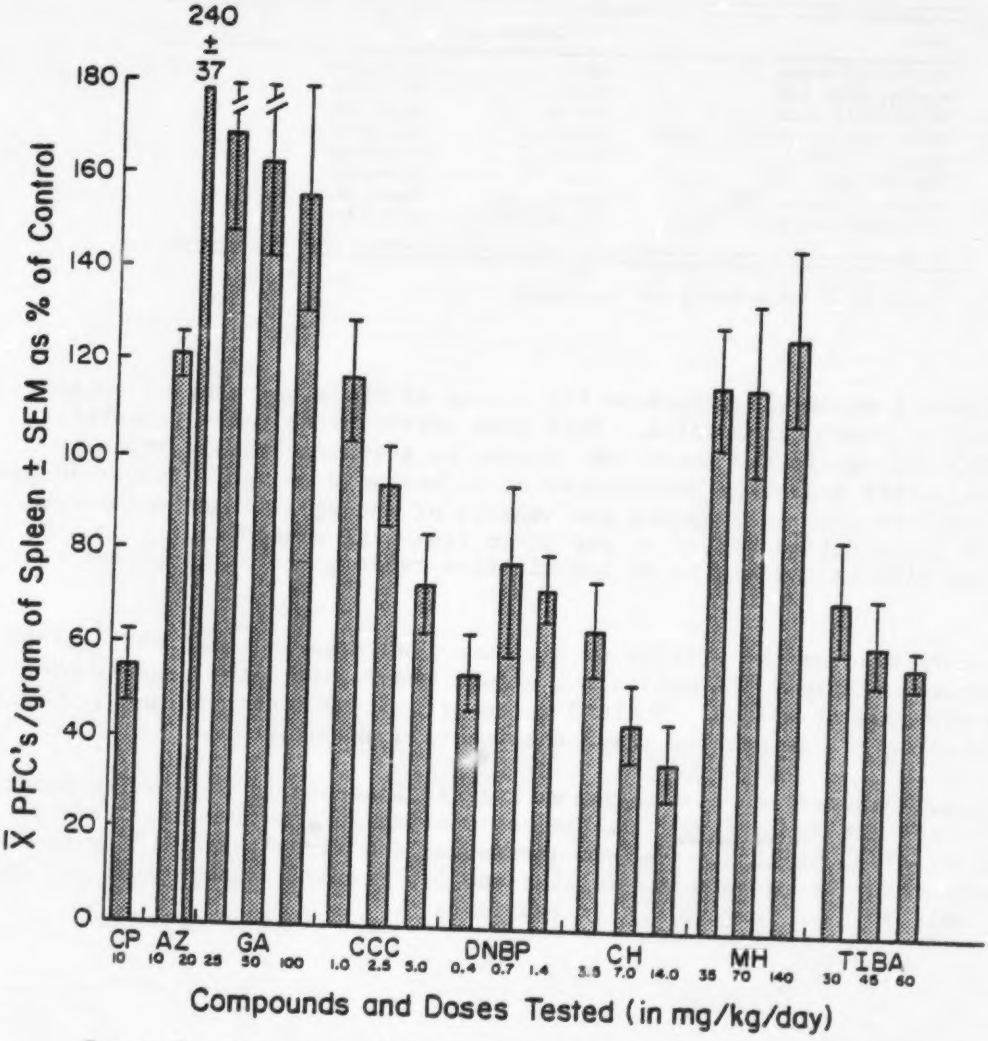

Figure 5. Effects of PGR's on Plaque Forming Cells (PFC's)/Gram of Spleen ± SEM, Expressed as a % of the Control (n=10).

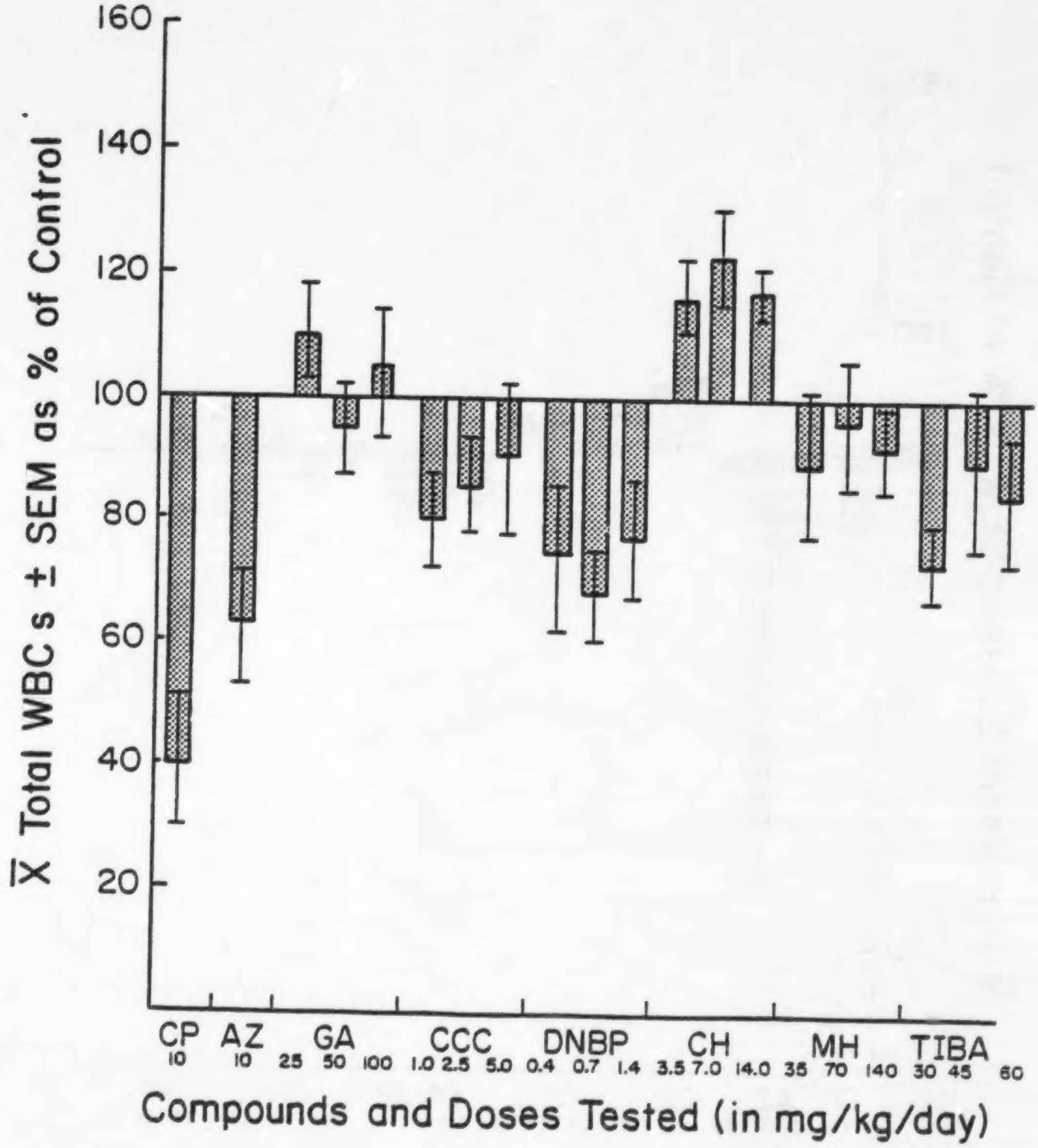

FIGURE 6. RESULTS OF PGR EFFECTS ON $\bar{X}$ TOTAL WHITE BLOOD CELL COUNT (WBC)±SEM, EXPRESSED AS A % OF CONTROL (N=10).

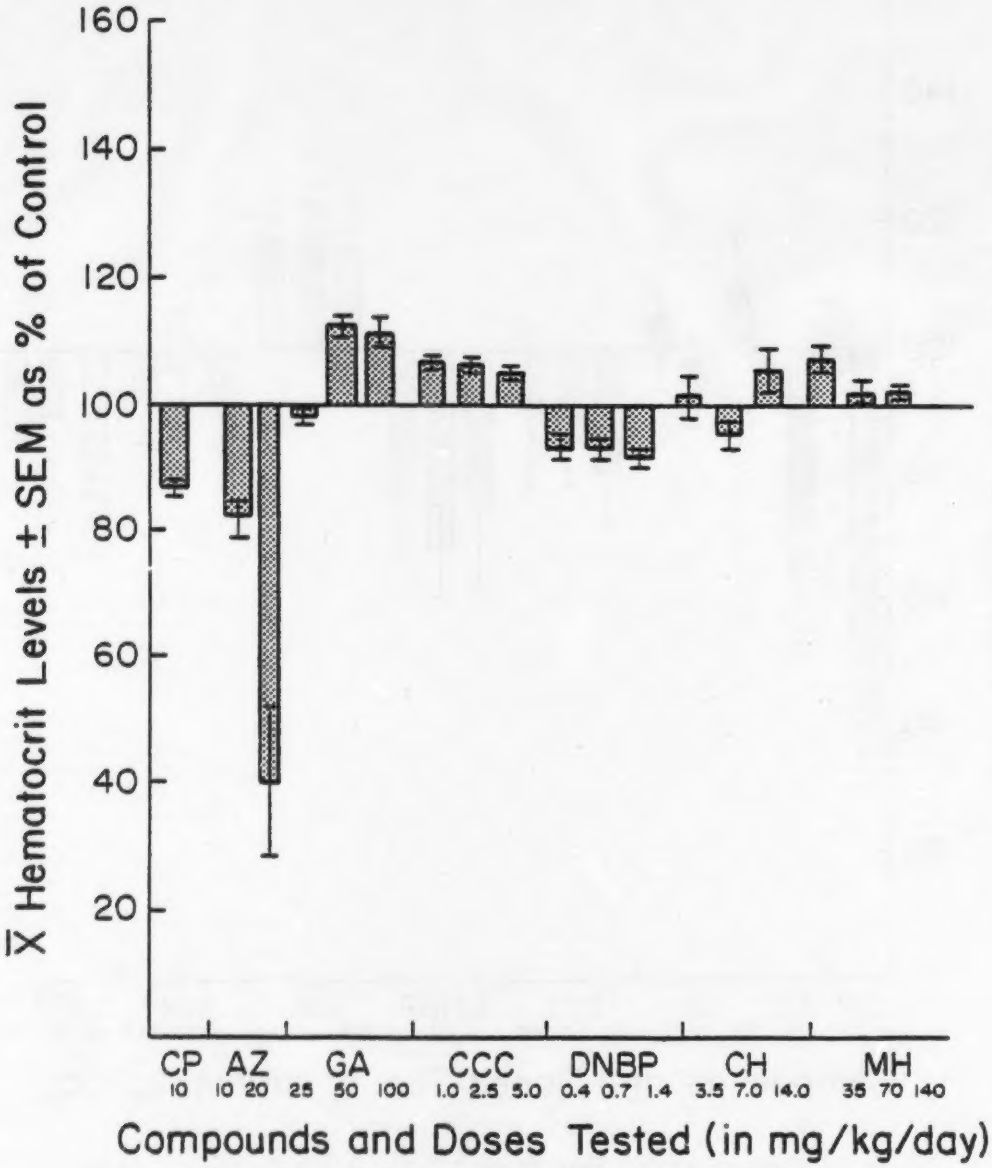

FIGURE 7. PGR EFFECTS ON $\bar{X}$ HEMATOCRIT LEVELS ± SEM AS A % OF CONTROL (N=10).

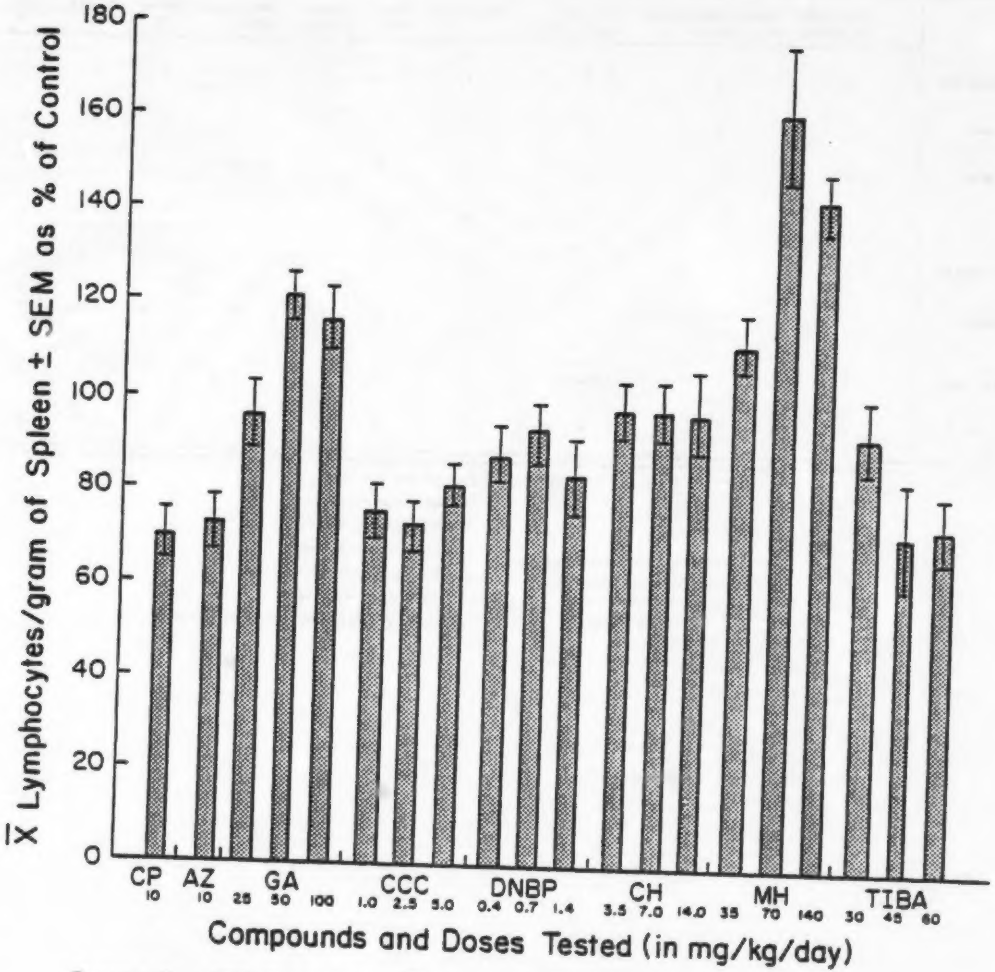

FIGURE 8. PGR EFFECTS ON $\overline{X}$ TOTAL LYMPHOCYTES/GRAM OF SPLEEN, EXPRESSED AS A % OF CONTROL (N=10).

TABLE 3. SUMMARY OF NET AFFECTS OF TEST COMPOUNDS ON VARIOUS PARAMETERS

COMPOUNDS	TEST PARAMETERS										
	PFC's/gm of spleen	Total lymphocytes/ gm of spleen	PFC's/10^6 viable cells	Hematocrit	Total WBC	Plasma protein	Hemolysin titer	Thymus Wt.	Spleen Wt.	Liver Wt.	Body Wt.
Cyclophosphamide	↓↓	↓	↓↓	↓	↓↓	0	↓↓	↓	↓	↑	0
Azathioprine	↑↑	↓	↑	↓↓	↓	-	↓	↓	↓	0	0
Cycloheximide	↓↓	0	↓↓	0	↑	0	↓↓	↓↓	0	↑	0
Dinoseb	↓	0	↓	↓	↓	-	-	-	0	0	0
Maleic Hydrazide	↑	↑	↓	0	↓	-	↓	↓	0	0	0
Triiodobenzoic Acid	↓	↓	↓	-	↓	-	↓	↓	0	0	0
Gibberellic Acid	↑	↑	variable	↑	↑	0	-	0	0	0	0
Chlorcholine Cl	↓	↓	↓	↑	↓	↓	-	0	0	0	0

Legend: ↑↑ strong elevation
↑ moderate elevation
0 no apparent affect
↓ moderate suppression
↓↓ strong suppression
- data not available
(significance levels not yet determined)

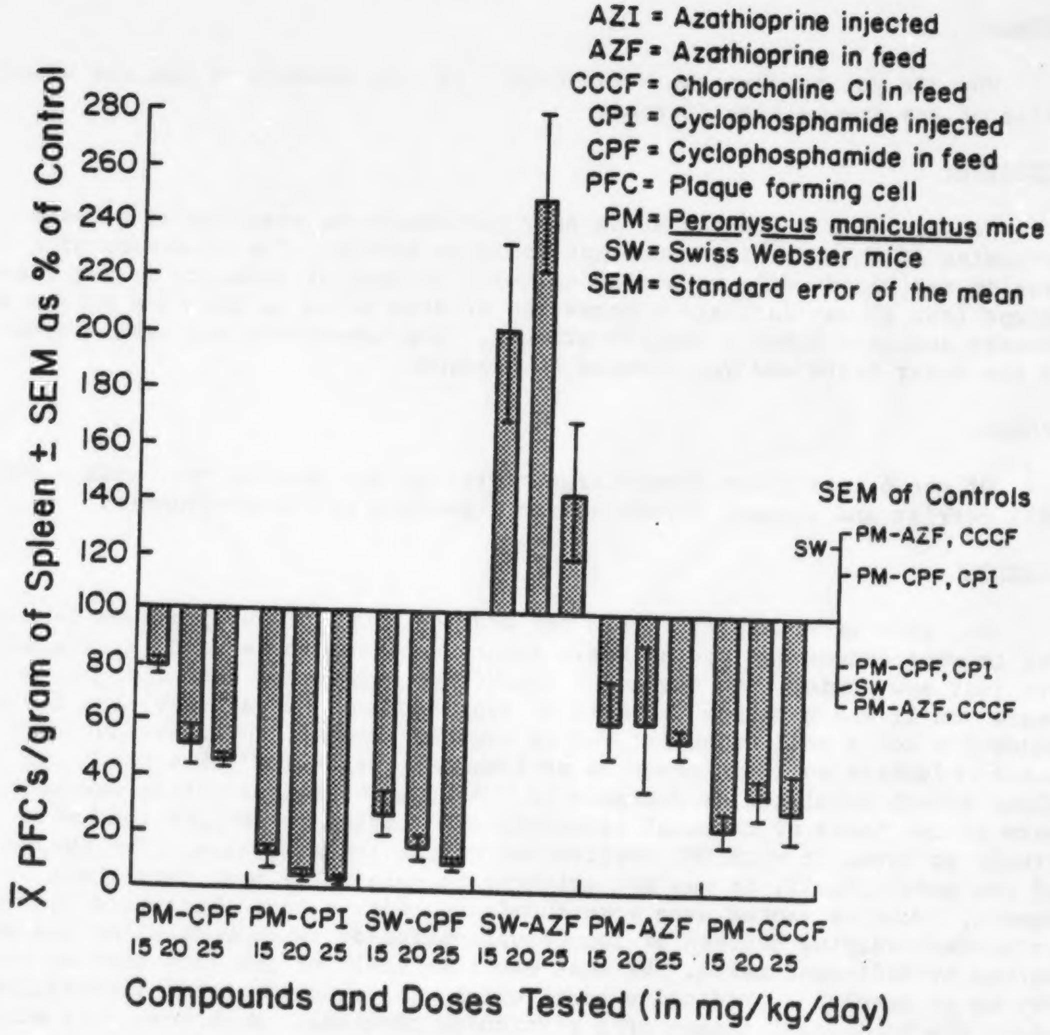

FIGURE 9. MEAN PFC'S PER GRAM OF SPLEEN FOR WHITE MICE AND PEROMYSCUS EXPOSED TO VARIOUS TOXICANTS.

QUESTIONS TO DR. HINSDILL (DR. ZEEMAN, DISCUSSANT LEADER)

ZEEMAN:

Why did you measure the hematocrit? Is this measure of the red blood cells of any immune significance?

HINSDILL:

We were simply looking at as many parameters as possible as we were screening so that nothing important would be missed. The relevence of a drop in red blood cells to either cellular or humoral immunity is not clear, except that it may indicate suppression of stem cells in the bone marrow and thereby suggests other potential effects. The hematocrit was easily added to the other tests and represented no hardship.

ZEEMAN:

Of the 6 test plant growth regulators, do you know to what extent they will persist and perhaps accumulate in organisms in the environment?

HINSDILL:

No, that is something we did not address. One of our problems is knowing to what extent any one of these agents is going to be used. These agents are just now coming into use and I think that within the next five or ten years you'll see a marked increase in application. It was very hard for me, since I'm not a soil scientist nor an organic chemist, to figure out what kinds of factors would interact to prolong the "half-life" of a particular plant growth regulator or decrease it. So the initial selection was made more on the basis of chemical structure and whether we thought they were likely to interact with DNA replication or the immune system. For purposes of the model itself, it was not critical to select the most persistant agents. What we wanted were some chemicals that we could plug into the model to produce varying degrees of immunosuppression or immunomodulation and which worked by different means. We must not lose sight of the fact that we are trying to develop a predictive model which will give important information about the biological impact of a particular chemical. Much more work will be required to answer questions regarding persistance and bioaccumulation of the plant growth regulators under a variety of environmental conditions.

KOCAN:

What effects other than immunosuppression are known to result from exposure to the test compounds you propose to use? Do any of the compounds you are using require metabolic activation by the mice?

HINSDILL:

There have been a number of studies carried out with some of them. Maleic hydrazide, for example, has been looked at for its ability to produce carcinogenesis and changes in DNA synthesis. There is some confusion when you go back and read the literature, because the results are dependent on the type of test system used. It was difficult to reach conclusions as to their biological impact. I presume that the usual kinds of safety testing were done by the parent chemical companies before they marketed the product, but you don't see much of this in the literature; again, it is very hard to determine what they found or looked for in the two year or similar feed study. We were primarily concerned with the immuno-suppressive ability of these compounds rather than liver toxicity or what they would do in an Ames mutagenicity test, but there is something in the order of 75 to 80 papers that have been published concerning other biological effects of these same chemicals.

FISHER:

Since certain immunosuppressants used in chemotherapy destroy platelets and can lead to chronic internal bleeding, is this type of stress and response being examined in your studies?

HINSDILL:

No, but none of the agents used to date have produced any overt internal bleeding detectable at the time of posting.

FISHER:

The reason I asked that question is that some of the other parameters that you will be measuring might affect the metabolism under certain conditions, production of the enzymes required to metabolize inactive forms of these agents might actually be shut off and thus the toxic effects would not be observed.

HINSDILL:

Activation of potentially dangerous chemicals can be very important. This is why we are doing feed studies primarily to make sure that the opportunity for activation in animals is present. There is a flaw and I will admit it. Maleic hydrazide, for example, if tested by the Ames mutagenicity test is not mutagenic, but if you first activate it with plant enzymes, it is mutagenic in this test. Now I don't know where plant activation comes into the picture, but it is obviously something we haven't built into the model yet. You can certainly envision a situation where the plants would take up the agents and activate them and then animals would eat the plants and get the fully activated agents.

Comments on
Immunotoxicity in Peromyscus maniculata

Maurice G. Zeeman
Food and Drug Administration

I was asked by Dr. Taub to briefly discuss why Dr. Hinsdill's portion of this three-part research project was worthy of interest and support by the Environmental Protection Agency. I will attempt to do so by first digressing for a moment into the subject of comparative immunology, then by returning to Dr. Hinsdill's part of this entire project, and finally by briefly discussing this research in relation to the entire project.

Around the turn of the century, the Russian researcher, Elie Metchnikoff, wrote a book while working at the Pasteur Institute in Paris. In this book, Metchnikoff focused attention on the fact that phagocytic cells were present and active inside all kinds of animals in the environment, from water fleas to goldfish. He postulated the value of these cells in resistance to disease. These phagocytic-type cells now turn out to be a very basic part of an immune-system that appears to be universal throughout multicellular animals. Something very much like a cell-mediated immune response has been discerned in most of the invertebrate phyla. In addition, basic research over the last 10-15 years has demonstrated that mammalian-like cell-mediated and humoral immune systems have been found to extend throughout all of the vertebrates.

Enough about comparative immunology, now back to Dr. Hinsdill's work and the EPA. I have been aware of Dr. Hinsdill's interest in Immunotoxicology for about three or four years now. His previous research on the PCB's and dioxins in lab rodents was of interest to me because I was conducting research on a persistent chlorinated hydrocarbon pollutant and looking for its effects on the immune system of organisms in the environment.

Over the last decade, Federal support of research on Immunotoxicology has often appeared to focus on research that had direct applications to man and/or his economically important food species. To me, support of Dr. Hinsdill's type of project by EPA indicates the recognition by a Federal agency that organisms in the environment do have immune systems that can be compromised by toxic agents. It is refreshing to realize that there exists in the EPA an awareness that organisms in the environment have immune systems which are probably just as essential to their health and survivial as our immune systems are to our own health and survival.

It is becoming more and more obvious that (under various conditions) environmental contaminants can compromise some elements of the complex (or simple) immune systems of animals residing in a contaminated environment.

Dr. Hinsdill's research has just started to examine the effects of a group of six potentially widely used plant growth regulators on a variety of humoral and cell-mediated immune parameters of the deer mouse, a rodent species widely distributed in nature. Attempts to make the chemical exposures as much like those expected in the field will eventually make this approach as natural as possible, given the constraints of a laboratory-type setting.

Dr. Hinsdill properly emphasizes that very little information is available on the potential toxicity of these compounds. Some of this information is summarized in publications such as the NIOSH Registry of Toxic Effects of Chemical Substances (1980) and in the Merck Index (1976). But toxicity is just one of the relevant characteristics of these chemicals that must be determined. How such chemicals act in the environment can be very important in assessing their potential for affecting various animals living in that environment. Are these chemicals capable of transport throughout the environment? Are they persistent? Do they bioaccumulate? DDT is not very acutely toxic to most mammals and birds, yet does appear capable of causing some degree of immunosuppression in several vertebrate species. The persistence and bioaccumulation potential of DDT would appear to endow this chemical (and other chemicals with similar properties) with considerably more potential for long-term impact than does the acute toxicity. Knowing that the plant growth regulator dinoseb is not tightly adsorbed to agricultural soils and can leach into rainwater (Herbicide Handbook Committee, 1979), but that it also appears to degrade quite rapidly in soil and on plants (Brown, 1978; Herbicide Handbook Committee, 1979) is probably just as relevant to evaluating dinosebs potential for environmental effects as is knowing it is highly toxic to several species of fish (Herbicide Handbook Committee, 1979; Johnson and Finley, 1980) and only moderately toxic to several bird and mammal species (Herbicide Handbook Committee, 1979). All such characteristics of chemicals may be of value in a first determination of which specific chemicals have the most potential for initiating effects (such as immunosuppression) in organisms in the environment.

In their research proposal, Dr. Hinsdill and the other investigators briefly discussed the practical and theoretical environmental significance of their overall project. The following three ideas on environmental contaminants and the potential value of the project seemed worthy of repeating.

First, this research could provide the basis for rapid screening of the effects of toxicants in the context of climate and disease, both common parts of natural systems. Second, this research should allow predictions of the consequences of environmental contamination on populations of animals at different times of the year and different latitudes and elevations. And lastly, this project could assist in reclaiming environments that have been mined or otherwise contaminated, since the necessary climates and quantities of food and water needed for persistence of species in a given area at all times of the year could then be estimated.

Dr. Hinsdill's project is only one of three parts, all of which will finally be integrated and synthesized into a model that will be useful in examining the interrelated effects of climate, disease organisms, and environmental chemicals on the growth and reproduction of a natural rodent population. I support the concept of such a research project, am pleasantly surprised at the volume of data generated in the six-month span this project has been funded, wish the researchers continued luck in their work, and finally applaud the EPA's vision in funding this type of research project.

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NEW APPROACHES TO MEASURING THE BIOLOGICAL IMPACT OF ENVIRONMENTAL TOXICANTS

III. HOST-PATHOGEN-TOXICANT INTERACTIONS: A PROGRESS REPORT

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ABSTRACT

The interactions between host, pathogen, and toxicant were examined as part of a study to determine the effects of physiologic stresses on growth and reproduction of deer mice (Peromyscus maniculatus). Breeding colony conditions are described. Two arboviruses which cause acute infections, Northway virus and Venezuelan equine encephalitis virus (VEEV), were tested for pathogenicity in deer mice. Northway virus caused no mortality, no viremias, and a weak ($\leq$1:20) antibody response. VEEV caused significant mortality (57%) at high doses and scattered mortality in all doses. Measurable viremias ($\geq$1:10) and antibody responses were present at all doses. An ID_{100} and an ID_{50} were determined. The effect on the host response to VEEV infection in mice continually fed 20 mg/kg of mouse of cyclophosphamide was examined. Mortality rates were increased with longer survival times, viremia onset showed no change, the duration of the viremia was lengthened, and peak viremia titers increased.

MOUSE BREEDING COLONY

The colony is housed in a separate room in the semi-isolation facility at Charmany Experimental Farms. The mice are kept in opaque polypropylene boxes with stainless steel tops and wood shavings as bedding. They are fed standard rodent lab chow and water *ad lib*. The light cycle is maintained at 14 light:10 dark and temperatures are approximately 23°C. No attempt is made to control humidity. Mice are weaned at 3 weeks and separated by sex. They are paired for breeding at 60 to 90 days of age.

It was originally thought that maximum reproduction could be obtained through the use of triad matings, one male and two females. However, discussion with other investigators and personal experience has shown that pair mating is more efficient for producing the maximum numbers of litters per month. Leaving the male with the female at all times assures breeding at the post-partum estrus, thus producing a litter every 3.5 weeks.

We presently have 100 breeding pairs and expect to maintain this number to assure a constant supply of experimental animals and replacement breeders. Breeding success has markedly improved since switching to pair mating and with the onset of spring (Figure 1). We are maintaining a continual cull and replacement process to achieve highest possible success. An attempt is made to keep inbreeding to a minimum and wild-caught pairs are periodically introduced to the colony to increase genetic heterogeneity.

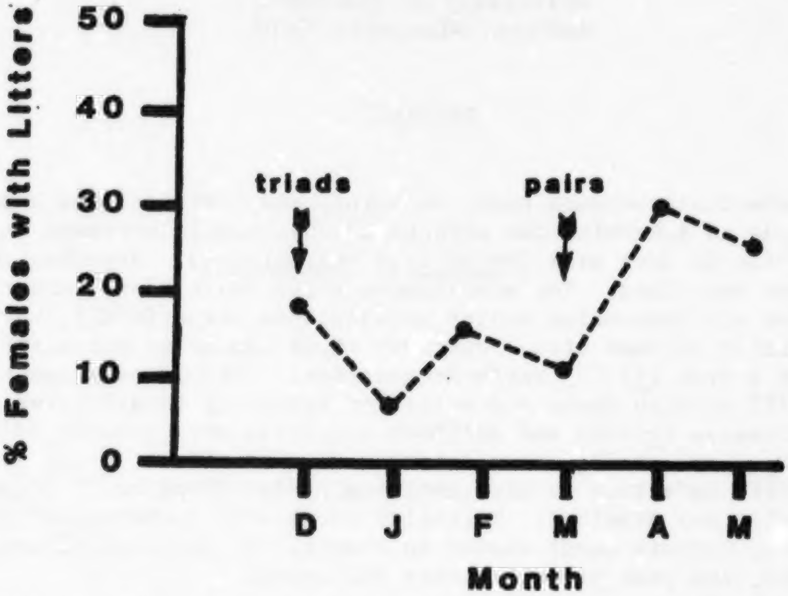

Figure 1. Breeding success of deer mice kept as triads (December 1980 - March 1981) and pairs (March - May 1981).

Dr. Glicken is in charge of medical surveillance of the colony. Any animals dying from other than experimental causes are necropsied and appropriate samples are taken for histopathology, bacteriology, and virology. Records are maintained for all necropsies. So far, we have seen one 3 year old female with a uterine prolapse and subsequent uremic syndrome due to blockage of the urethra, and two old females with extensive mammary tumors. One of these mice also had cerebral hemorrhage which had caused central nervous system signs prior to euthanasia. Interestingly, there appears to be no previous reports in the literature of deer mice with mammary tumors.

Health screens have also been performed to establish what potential pathogens are already present in our colony. In addition, all animals entering our breeding colony are bled and a serum sample is stored for future reference in the event of a major disease outbreak. Serological tests have shown the mice to be free of antibodies to 11 viruses commonly found in laboratory mice (Table 1).

TABLE 1. VIRUSES COMMONLY FOUND IN LABORATORY MICE

Polyoma virus
Reovirus, type 3
Mouse poliovirus, strain GD VII
Ectromelia
K-virus
Sendai virus
Mouse adenovirus
Mouse hepatitis virus
Lymphocytic choriomeningitis virus
Pneumonia virus of mice
Mammary tumor virus of mice

However, 20 pairs of mice were recently received from the colony of Dr. C. Terman, College of William and Mary, VA to help expand our colony. These mice have low level antibody titers (1:10) to Sendai virus. Bacteriological cultures of organ swabs and tracheal washings were negative for *Mycoplasma* spp. and positive for *Staphlococci* spp. (lung, liver), *Streptococci* spp. (tracheal wash) and *Corynebacterium* spp. (liver). These bacteria were encountered in small numbers and their presence was not associated with any lesions. All mice were negative for internal parasites. Health screens still need to be done on a representative sample of the wild-caught mice. Generally, we feel that we have a very healthy colony.

The importance of this aspect of the work cannot be overstated. These mice are quite different than normal laboratory white mice in terms of growth and reproduction. Any serious viral or bacterial outbreak in one or more of the colonies would seriously interrupt the breeding schedule and could, if not detected, influence experimental groups in the combined physiologic, toxic and pathogenic stress experiments.

HOST-PATHOGEN INTERACTION

Development of the model of the effects of physiologic stresses on growth and reproduction of deer mice will require establishment of several non-lethal bacterial and viral infections. The bacterial and viral diseases of this species have not been well studied. We wish to use at least two bacterial infections, four acute viral infections, and one chronic viral infection in the model. Each pathogen establishes its own relationship with its host, which may be affected to varying degrees by toxicants or environmental factors. Thus, the more pathogens used, the more useful the model will be. We recently did a field study in northern Alberta and have isolated a variant of Modoc virus from an apparently healthy deer mouse. Adult deer mice infected in the laboratory with this virus survived without apparent disease (1). We believe that Modoc virus can be used to assess effects of chronic viral infection. Rodent-associated (at least in part of their geographic range) viruses that will be used for acute infection include Northway, Klamath, *C. gapperi* agent, *Peromyscus* virus, western equine encephalitis, and the TC-83 (vaccine) strain of Venezuelan equine encephalitis virus. All but TC-83 were isolated from wild North American rodents and all are sufficiently safe to handle in the laboratory.

PATHOGEN SELECTION

The first virus selected for testing was Northway virus. Little is known about this virus. It was the first arbovirus of the Bunyamwera group to be found in Alaska (2) where the original isolation was from mosquitoes (*Aedes* spp.) and sentinel rabbits (*Oryctolagus cuniculus*). McLean, et al (1978) (3) at the University of British Columbia have been studying the transmission of the virus by the Arctic mosquitoes, but no work has been done on its infectivity and pathogenicity in mammals.

Stock virus was grown from seed stock obtained from Dr. Calisher, Center for Disease Control, Fort Collins, Colorado. Two separate lots of Alaska-origin virus were passed in suckling mouse brains. This was only the second passage of the field isolation in a vertebrate so there is little likelihood that the virus changed very much from its original form. Both lots were titered in cell culture by a standard plaque assay. Lot 1 had 2.2×10^6 plaque forming units (pfu) per ml and Lot 2 had 1.0×10^7 pfu/ml. Thus, infectivity of the second lot was about 10-fold greater than the first.

The virus identity was confirmed with reference antiserum obtained from the Center for Disease Control, utilizing the plaque-reduction test. A working stock of hyperimmune mouse ascitic fluid produced for future reference work titered $1:10^4$ against 100 $TCID_{50}$ of virus.

Two experiments were performed to find a dose and route of virus inoculation that would infect 100 percent of the mice while killing none. In the first experiment, the first lot of virus was serially diluted 10-fold from 10^{-1} to 10^{-5}. Each of six mice per dilution were inoculated subcutaneously (SC) with 0.2 ml. They were bled daily for 7 days to collect whole blood for virus isolation and then bled every 3-7 days for 4 more weeks to collect serum for antibody assay. Since there were no mortalities other than those due to the trauma of bleeding, only the lowest dilution group (10^6 pfu/ml) was tested for the presence of viremias and antibody response. Testing of the whole blood for virus was attempted in the microtiter cell culture system, but the red blood cells made the cell cultures difficult to see. Therefore, the blood was assayed for virus by intracranial inoculations of suckling mice. Brain suspensions of any dead or sick suckling mice were tested in cell culture to confirm the presence of virus. Only 1 of the 6 deer mice had a measurable viremia--with a titer of 2.3 $\log_{10}$. Sera tested by microtiter serum neutralization (SN) showed an antibody response in all 6 mice on days 8, 14, and 28 post inoculation.

The second lot of virus was injected SC as well as intraperitoneally (IP), in an effort to achieve more rapid adsorption along with a depot for slow release to maintain virus levels. Since Lot 2 had 10-fold more virus than Lot 1, higher dosages were attained. Serial 10-fold dilutions were made and injected into 7 mice per group. The same bleeding schedule was followed as for Lot 1. Once again there were no mortalities so only the lowest dilution group was tested. Whole blood diluted 1:10 was tested in the microtiter cell culture system for the presence of virus without the problem of visibility. Again, no viremias were found. Sera from mice sampled on days 9, 14, and 20 post inoculation were titrated in the SN test and found to have maximum titers of 1:10 for days 9 and 14 and 1:20 for day 20.

We concluded that Northway virus is not very pathogenic for deer mice although the mice apparently become infected and are able to mount an antibody response to it. It may be that deer mice are not natural hosts for this virus, even though their range includes the parts of Alaska and Yukon Territory where the virus has been found. Rather than trying to adapt the virus by repeated passage in deer mice we considered it to be more advantageous to work with another virus that is known to be more pathogenic. We sought a virus that would consistently induce a measurable viremia in deer mice to determine if that viremia would be increased or decreased when the mice were subjected to the stresses of toxicants and climatic factors.

The next virus selected for testing was another arbovirus, Venezuelan equine encephalitis virus (VEEV). Wild deer mice are infected by VEEV (4) and deer mice infected in the laboratory produced good viremias and antibody responses (5,6). These previous studies used a field strain of virus which resulted in high rates of mortality as well. Therefore, we elected to use the vaccine strain of the virus to find an infectious but non-lethal dose. In addition, this strain is of lower pathogenicity to humans and can be used in all phases of the study with minimal risks and handling restraints.

A working stock of virus was grown in VERO cells and the usual virological standardization tests were performed. The working stock had an infectivity of 8×10^8 pfu/ml and a tissue culture 50% infective dose ($TCID_{50}$) of 5.58 $\log_{10}$. Suckling mouse intracranial inoculations resulted in a $SMICLD_{50}$ of 4.83 $\log_{10}$. Thus, the virus is about 10 times more pathogenic for suckling mice than for cell cultures. A positive plaque reduction test with reference VEEV antiserum verified the identity of the virus and a supply of rabbit hyperimmune serum was produced for future reference.

The virus was serially diluted 10-fold to contain 8 x 10^7 to 8 pfu/ml. Five to 10 mice per dilution were each inoculated SC and IP with a total dose of 0.4 ml. This dose caused mortality in all dilutions except 8 pfu/ml (Table 2a). Tissue culture microtiter tests showed viremias in 100 percent of the mice in dilutions 8 x 10^7, 8 x 10^4, 8 x 10^3, and 8 x 10^2 pfu/ml (8 x 10^6 and 8 x 10^5 were not tested). Eighty percent of the mice in the 80 pfu/ml dilution had measurable viremias ($\geq$1:10) as did 50 percent of those in the 8 pfu/ml dilution. Antibody titers were determined at selected intervals from 8 to 39 days post inoculation for mice receiving dilutions 8 x 10^4 and 8 x 10^3 pfu/ml (Table 3). VEEV antibodies were present beginning on day 8, with titers ranging from 1:10 to 1:80, with peaks on days 19 to 24.

TABLE 2. NUMBERS OF DEER MICE (P. MANICULATUS) DYING POST INOCULATION WITH 0.4 ML SC AND IP OF VEE VIRUS STRAIN TC-83

a. No cyclophosphamide in feed.

Virus dilution (pfu/ml)	Days post inoculation								Total number		%
	7	8	9	10	...	18	20	21-62	Dead	Total	Dead
8 x 10^7	1	1		1		1			4	7	57
8 x 10^6		2							2	6	33
8 x 10^5	3								3	7	43
8 x 10^4							1		1	7	14
8 x 10^3		1*							1	7	14
8 x 10^2	1								1	10	10
80		1							1	10	10
8									0	8	0

b. Cyclophosphamide in feed, 20 mg/kg mouse.

Virus dilution (pfu/ml)	Days post inoculation								Total Number		
	7	8	9	10	11	12	17-21	31	Dead	Total	Dead
8 x 10^4	1	1		1		1			4	10	40
8 x 10^2									0	10	0
80	1						2		3	10	30
8		1					1†	1	3	10	30

* Virus isolated from brain
† Missing, presumed dead

TABLE 3. MEAN* VIREMIA AND ANTIBODY TITERS IN DEER MICE (*P. MANICULATUS*) INOCULATED SC AND IP WITH 0.4 ML VEE VIRUS STRAIN TC-83

Days post inoculation	Virus dilution (pfu/ml)		
	8×10^7	8×10^4	8×10^3
		Viremia	
1	141†	91	46
2	89	16	2
3	141	33	4
4	43	3	0
5	0	0	0
6	0	0	0
7	0	0	0
		Antibody	
8	§	4	6
10	§	15	13
14	§	35	8
19	§	52	10
24	§	56	11
32	§	34	8
39	§	25	15
N	6	5	5

* Geometric means ($\bar{x}_g$)

§ Not measured

† Reciprocal of dilution

SC and IP inoculation of 0.4 ml of the 8×10^4 pfu/ml dilution of this working stock of virus was selected as the test dose for the mice in the physiological stress experiments. This dose infected all of the mice with measurable viremia titers and relatively high antibody production. It appears that some mortality can be expected at any dose (Table 2a), probably due to occasional "supersusceptible" mice that are unable to contain the infection regardless of the size of the initial dose. The 8×10^3 pfu/ml dilution also infected all mice but was not selected because the viremia and antibody levels were not as high as those in the 8×10^4 pfu/ml group.

An ID_{50} (infectious dose of 50 percent of the animals) was needed for use as the center replicate in the physiological stress experiment. This was readily shown to be the 8 pfu/ml dilution of the working stock (Table 4). Determination of antibody titers at this level is still in progress.

TABLE 4. PERCENT DEER MICE (P. MANICULATUS) WITH MEASURABLE VIREMIAS (≤1:10) AFTER SC AND IP INOCULATION WITH 0.4 ML OF VEE VIRUS STRAIN TC-83 AND + OR -20 MG/KG MOUSE CYCLOPHOSPHAMIDE (CP) IN THE FEED

Days post inoculation	8 x 10^7		8 x 10^4		8 x 10^3		8 x 10^2		80		8	
	Control	+CP	Control	+CP	Control	+CP	Control	+CP	Control	+CP	Control	+CP
1	100	§	100	80	100	§	90	80	80	50	20	30
2	100	§	60	80	33	§	70	90	70	70	30	70
3	100	§	80	90	50	§	80	80	70	90	40	33†
4	100	§	20	50	17*	§	90	90	60	60	30	44†
5	0	§	0‡	40‡	17*	§	10*‡	30‡	10*‡	30‡	10	22†
6	0	§	0	20*	17*	§	§	0	§	20	§	10
7	0	§	0	20*	17*	§	§	0	§	0	§	0
Total % viremic	100	§	100	100	100	§	100	90	80	100	50	90
N	6	§	5	10	6	§	10	10	10	10	10	10

* Died

† N = 9

‡ Significant difference between +CP and Control percentage (Student's t-test P≤0.05)

§ Not measured

PATHOGEN-TOXICANT INTERACTION

A review article by Nathanson and Cole (1971) (7) discusses the effects of immunosuppression caused by a single injection of cyclophosphamide (CP) on acute virus infections in mice. In summary, viremia titers, mortality rate, and survival time were all increased. Work done in a collaborative project in Dr. R. Hinsdill's laboratory (8) has shown that continuous feeding of lower levels of CP can also result in immunosuppression; specifically, a decrease in number of circulating lymphocytes and antibody-producing cells in the spleen. Since arbovirus infections are terminated by the production of neutralizing antibodies (9) it was necessary to determine what effect this chronic low level of CP in the feed would have on a sublethal infection of VEEV in the deer mice. An appropriate adjustment in virus dose could then be made, if necessary, prior to using these compounds as stressors in the physiological experiment.

Forty mice were fed CP at 20 mg/kg mouse for 23 days. Groups of mice were then injected SC and IP with 0.4 ml per mouse of VEEV at 8×10^4, 8×10^2, 80, or 8 pfu/ml dilutions (10 mice per dilution). Ten control mice were fed feed mixed with saline instead of CP and inoculated with the same dilutions of virus plus one group inoculated with diluent only. The 40 experimental mice were fed CP-feed for the remainder of the study. Day 23 was chosen for inoculation as this is the time used for antigen stimulation in the prior CP studies done in Dr. Hinsdill's laboratory. Viremias and mortality rates of the mice fed CP were compared with those of the mice in the experiment with VEEV alone, described above.

Table 2b shows the mortality pattern for mice injected with VEEV while being fed CP. A comparison with Table 2a shows that feeding CP results in higher mortality rates and increased survival time.

The time of onset of viremia was not affected by CP feeding. The percentage of mice with viremia onset on Day 1 was not significantly different (Student's t-test, $P \leq 0.05$) between mice fed CP (CP treated) and mice not fed CP (controls) for any virus dilution (Table 4). However, the duration of viremia was increased as a result of CP feeding. Viremias lasted for a maximum of 4 days in the control mice whereas the CP treated mice had viremias persisting for up to 5 days for all virus dilutions except 80 pfu/ml, where the viremia persisted for a maximum of 6 days (Table 4).

The geometric mean ($\bar{x}_g$) peak viremia titer for each virus dilution is shown in Figure 2. It is evident that CP significantly ($P \leq 0.05$) increased the peak viremia titer, except for the 8×10^4 pfu/ml dilution of virus. The reason for this discrepancy is unknown.

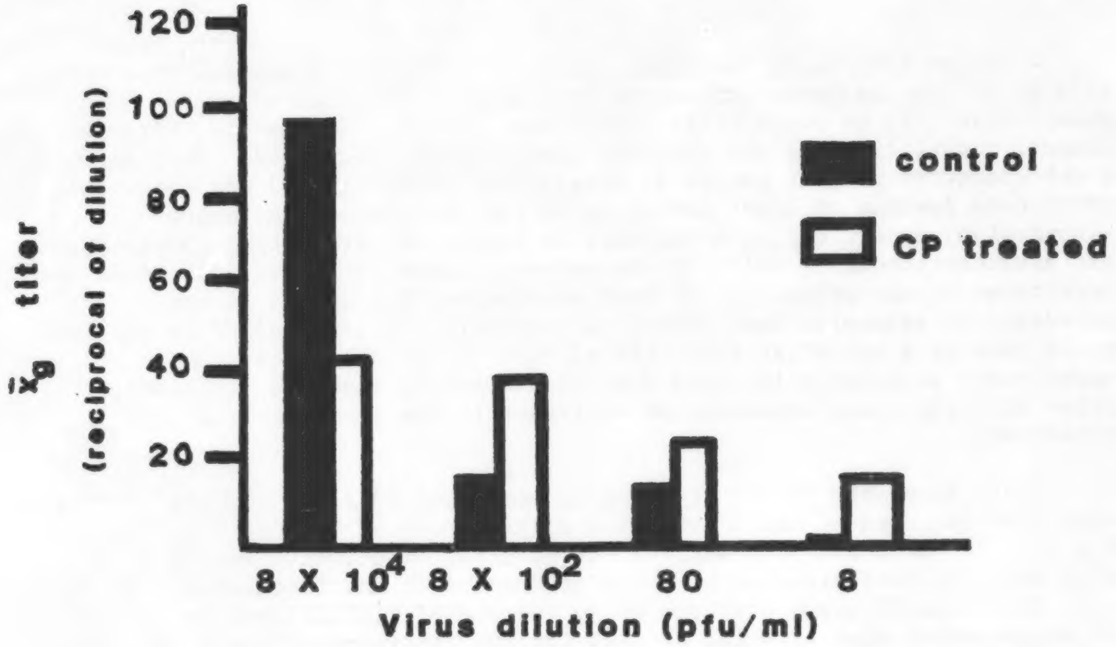

Figure 2. Mean ($\bar{x}_g$) peak viremia titers of deer mice (*P. maniculatus*) inoculated SC + IP with 0.4 ml of VEE virus strain TC-83 and fed + or -20 mg/kg mouse cyclophosphamide (CP).

In summary, chronic low levels of CP in the food has an immunosuppressive effect on deer mice. This causes an acute, sublethal infection of the arbovirus VEEV to attain higher viremia levels of longer duration and results in increased mortality rates. The effects of CP on the antibody response to an acute infection is still being evaluated.

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Comments on
Host-Pathogen-Toxicant Interactions:
A Progress Report

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At the time of the presentation, this project had only been underway for nine months. Therefore, only the preliminary studies had been completed and much of the proposed research had not yet been initiated.

Mouse Breeding Colony

A breeding colony of deer-mice (*Peromyscus maniculatus*) has been established at the University of Wisconsin's semi-isolation facility in Madison, Wisconsin. The colony currently consists of 100 breeding pairs and the breeding success has been increased to 30% (up from 10%) since the number of mice per cage was reduced from three to two. This success also coincided with the beginning of spring. All of the animals have been or will be checked for the presence of pathogenic organisms and regularly monitored for signs of disease or parasites.

Host-Pathogen Interaction

Several strains of low virulence virus stock were tested in mice to obtain a non-fatal/high infectivity test virus for future studies with toxicant. The third strain tested (VEE vaccine) produced the desired combination of effects and was tested further.

Pathogen-Toxicant Interaction

To determine the combined effects of virus and toxicant (cyclophosphamide CP), two groups of mice were given inoculations of virus ranging from 8×10^7 to 8 plaque forming units per ml. One group was fed a diet of 20 mg/Kg mouse for 23 days prior to virus inoculation. The data collected after these treatments showed a definite supression of an antibody response to virus in the CP treated group as well as a higher virus titer. There was, however, some discrepancy in the mortality figures which showed all 10 animals surviving a virus dose of 800 pfu and CP, while animals receiving only 8 pfu's suffered a 30% mortality. Otherwise, the virus and CP group had a higher overall mortality rate than the virus only group.

This project seems to be off to a good start and with time should provide some very useful data concerning the combined effects of pathogens and toxic chemicals.

EFFECTS OF MILORGANITE ON TWO GRASSLAND ECOSYSTEMS

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INTRODUCTION

Numerous studies have been concerned with the effects of sludge on young (e.g., agricultural) or mature (e.g., forest) ecosystems (1, 2, 3). Few studies have focused upon the intermediate (e.g., old-field) stages of ecosystem development (4) as a feasible and efficient community-type for the disposal and recycling of sewage waste products. Such old-field communities are highly productive (5, 6, 7) and are efficient at recycling nutrients (8). We hypothesized in the present study that the intermediate stages of community development (i.e., the old-field or grassland meadows) may well be the best community-type for processing sewage sludge.

Previous sludge studies have been mainly concerned with the lower levels of biological organization, i.e., with the cellular (9), the histological (10), and organismic (11) levels of organization. Most of these studies focused upon individual plant or animal species, or their respective component parts, without attempting to analyze the system as a whole (12). Odum (13) has pointed out that problems often arise when stressors (e.g., sludge) are tested at one level (e.g., individual) and then used without further study at another level (e.g., ecosystem). Also, by studying the ecosystem as a whole, man should be able better to predict the long-term response of stress or nutrient effects on natural ecosystems (14, 15, 16). Barrett *et al*. (17) outlined specific guidelines for testing stressors, such as sludge, at the ecosystem level. Thus, another major objective of this investigation was to identify indicator "white mice" indices (17) regarding the effects of sludge on total ecosystems. Both structural (e.g., plant biomass and species diversity) and functional (e.g., productivity and developmental) parameters are being measured and evaluated within two different grassland (old-field) communities in order to recommend properly such indices for future studies.

At present, no studies have been conducted which simultaneously attempt (a) to measure, evaluate, and compare the effects of sludge applications on contrasting community-types treated in an idential manner; (b) to evaluate sludge effects on several different levels of biological organization; (c) to isolate indicator parameters which may be indicative of total stress response;

and (d) to identify the energy flow (fate) pathways of heavy metals (cadmium, zinc, lead, and copper) as they move from the sludge through the producer trophic level into the primary consumer (Microtus) small mammal populations functioning within these old-field communities. We hypothesizes that wild mammalian populations tend to depict better sludge effects (e.g., biological magnification) than by extrapolating strictly from laboratory populations.

BACKGROUND INFORMATION

The present long-term study was initiated in 1978 at the Miami University Ecology Research Center located on the Bachelor Wildlife Reserve near Oxford, Ohio. The initial phase (1978-1980) of the study was supported by a subcontract of EPA No. R 805445-01 entitled "Evaluation of the Health Risks Associated with the Treatment and Disposal of Municipal Wastewater and Sludge" awarded to the Department of Environmental Health, University of Cincinnati, Dr. C. Scott Clark, Principal Investigator. I served as Principal Investigator of the subcontract to Miami University. The present EPA Grant (R807370-01) became effective 13 May 1980. Thus, three (3) years of field data have been collected; only one (1) year of data has been collected concerning the present grant.

EXPERIMENTAL DESIGN

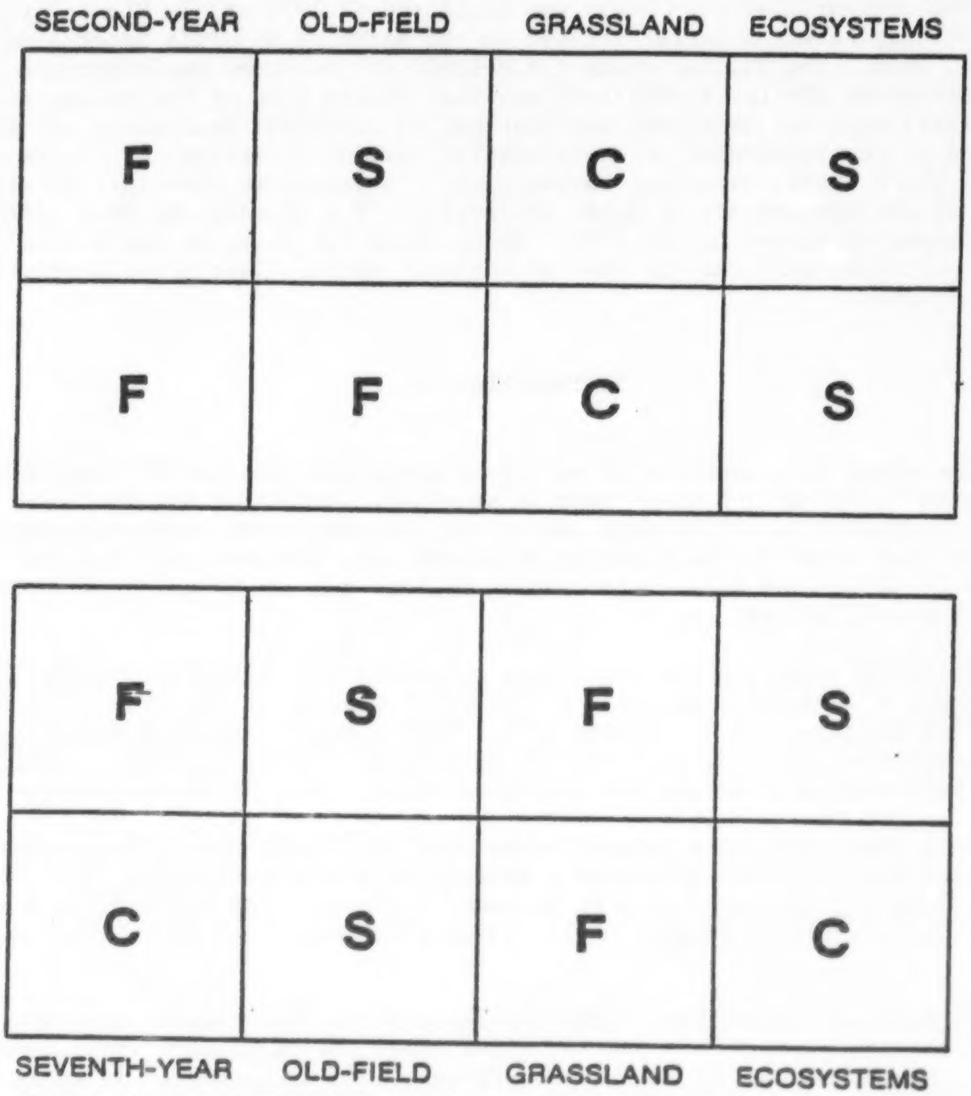
The study site consists of an 0.8-ha grassland (old-field) community presently (1981) in the third year of secondary succession and an 0.8-ha (old-field) community in the seventh year of succession. Each community-type was divided into eight 0.1-ha plots by enclosing each plot with 0.2 x 2.0 m sheets of galvanized steel. Enclosures of this type have been previously described (14, 16, 18).

All eight plots in the third-year old-field were originally plowed and disked and a commercial fertilizer (12-12-12) applied at the rate of 56.5 kg ha^{-1} (300 lbs/acre) on 24 October 1977. Winter wheat (Triticum aesveticum Ranger variety) was sown at a rate of 22.6 kg ha^{-1} on 25 October. These plots were then left fallow the following years, i.e., up to the present time.

In October 1974, the present seven-year old-fields were plowed, disked, and all eight (8) plots sown with a mixture of grasses consisting of 6.8 kg fescue (Festuca elatior), 11.3 kg Kentucky bluegrass (Poa pratensis), and 4.5 kg rye grass (Lolium perenne) (19). These plots have been left fallow since 1974.

In each community-type, three replicate plots were treated with dried sludge (Milorganite; 6-2-0, N-P-K), three replicate plots were treated with fertilizer (6-2-0, N-P-K), and two plots were left untreated to serve as controls (Fig. 1). Milorganite is a heat-dried, anaerobically digested sludge that has been commercially available since 1927 (20). Milorganite for each of the past three years (1978-1980) has been applied at an enrichment rate

EXPERIMENTAL DESIGN


SECOND-YEAR OLD-FIELD GRASSLAND ECOSYSTEMS
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SEVENTH-YEAR OLD-FIELD GRASSLAND ECOSYSTEMS

Figure 1. Experimental design for the proposed investigation. Treatments were selected at random within each community type. Each plot represents a quarter-acre (0.1 ha) area. Sludge and fertilizer will each be applied at monthly intervals from May through September in a 6-2-0 (N-P-K) ratio.

equivalent to 1792 kg/ha (1600 lbs/acre) monthly. Sludge is applied five times (May-September) per year. An equivalent amount of fertilizer is applied to each community-type the same time sludge is applied. The same design (i.e., the same application rates) is also being used this summer (1981). It is only from long-term studies of this nature that chronic effects of municipal sludge may be evaluated in a quantitative manner.

RESULTS

PRODUCER TROPHIC LEVEL

In 1978, no significant differences due to treatment were found in species richness in the annual (wheat) field or in the perennial (fourth-year old-field) field. There was a significant increase in productivity in both community-types in the fertilizer and sludge treatments; however, the increase was larger and occurred earlier in the fertilizer treatments. Estimates of net primary produtivity (NPP) in the agricultural field were 1690 g m^{-2} yr^{-1} ± 84 (SE) in the fertilizer treatment, 1240 g m^{-2} yr^{-1} ± 58 (SE) in the sludge treatment, and 772 g m^{-2} yr^{-1} ± 3 (SE) in the control. Estimates of NPP in the fourth year old-field were 1110 g m^{-2} yr^{-1} ± 39 (SE) in the fertilizer treatment, 684 g m^{-2} yr^{-1} ± 69 (SE) in the sludge treatment, and 424 g m^{-2} yr^{-1} ± 37 (SE) in the control. It appeared that the nutrient enrichment effects on plant community structure and function were greater in the agricultural field than in the old-field.

In 1979 and 1980, NPP in July was significantly higher ($P \leq 0.05$) in the one-year old-field fertilized and sludge plots as compared to control plots (Fig. 2). Interestingly, the two-year old-field control plots exhibited significantly higher ($P \leq 0.05$) values than the sludge plots, but not significantly higher than the fertilized plots. Only one significant difference in NPP was observed in the five-year or six-year old-fields; nutrient-enriched plots in the six-year old-field were significantly greater than control plots in July (Fig. 2).

Control plots exhibited significantly greater ($P \leq 0.05$) values for species richness and apportionment than the nutrient-enriched plots in the one-year and two-year old-fields. No differences in species richness or apportionment were observed in the five-year or six-year old-fields (Fig. 3). Competition for nutrients appeared to account for these differences. Differences in community function (e.g., NPP) were found to relate directly to changes in community structure (e.g., species diversity). Changes in plant species composition and NPP for 1978-1980 are summarized in Tables 1 and 2.

PRIMARY CONSUMER TROPHIC LEVEL

Meadow vole (Microtus pennsylvanicus) populations have been monitored for three years by live-trapping 16 0.1-ha enclosures. Vole population densities were affected by treatment only in the wheat plots in 1978 (Fig. 4). Sludge-treated wheat plots had significantly higher population densities than either fertilizer or control plots. However, survivorship, longevity, and percentage of breeding adults were not affected by sludge treatment. Sex

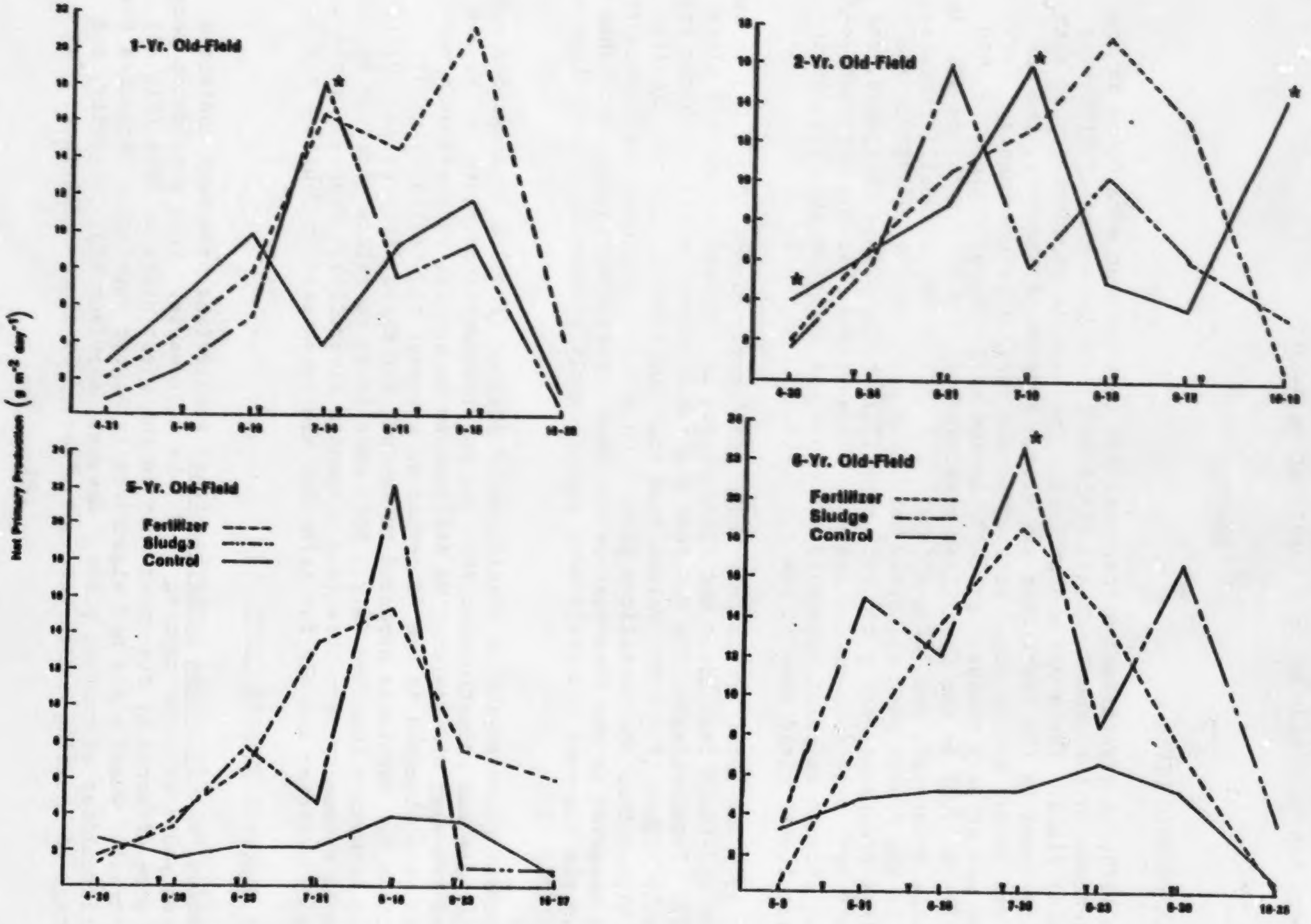

Figure 2. Changes in NPP (g m^{-2} day^{-1}) in 1979 and 1980 for both community types.

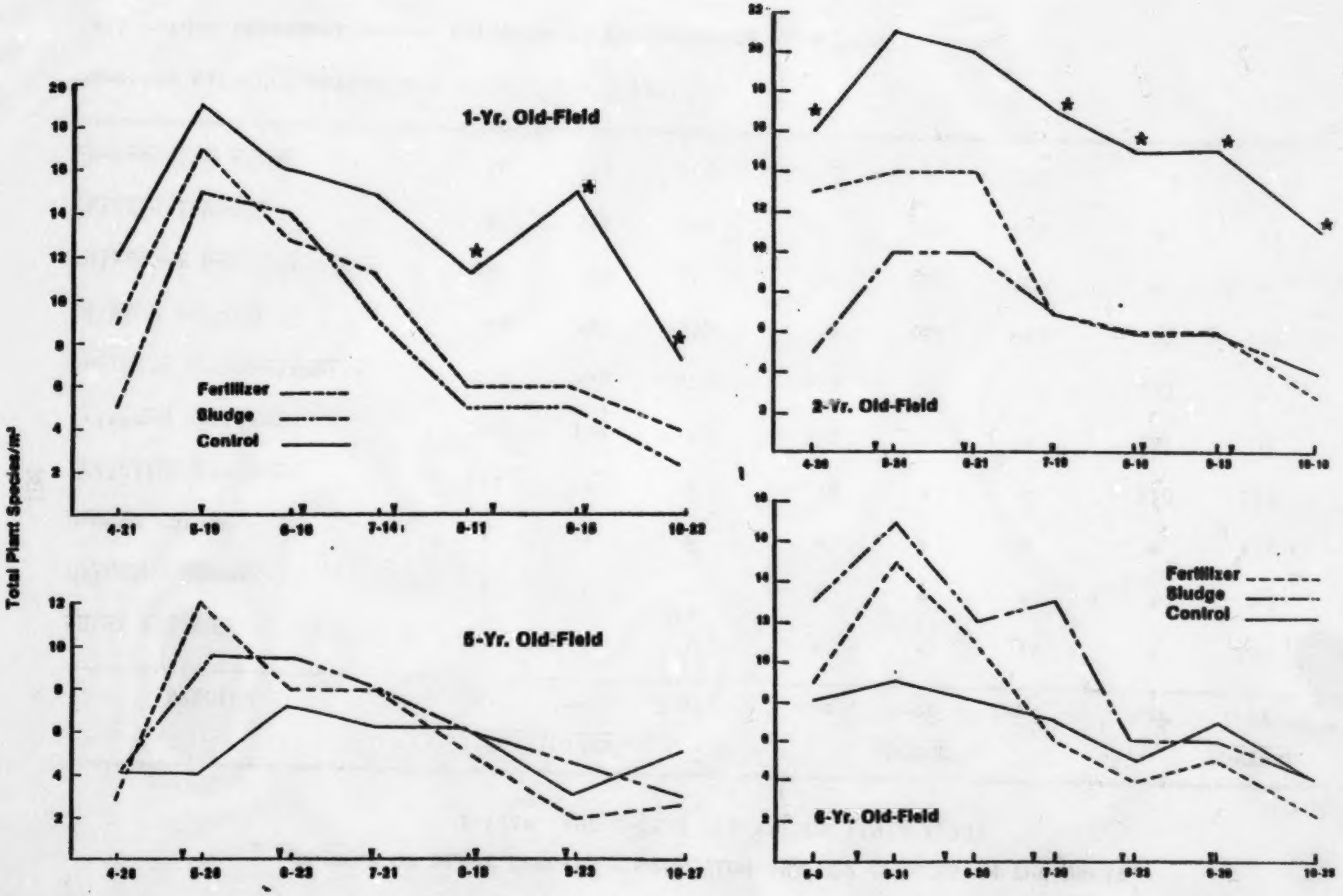

Figure 3. Changes in total plant species (number of species/m^{-2}) in 1979 and 1980 for both community-types.

TABLE 1

SUMMARY OF PLANT SPECIES COMPOSITION AND NPP CHANGES IN THE WHEAT, 1-YEAR, AND 2-YEAR OLD-FIELDS (1978-1980)

SPECIES	FERTILIZER			SLUDGE			CONTROL		
	Agr	1-yr	2-yr	Agr	1-yr	2-yr	Agr	1-yr	2-yr
Aster pilosus	*	43	*	*	*	24	*	199	840
Erigeron annuus	*	*	*	*	*	*	*	82	293
Daucus carota	*	*	*	*	*	*	*	176	*
Trifolium pratense	14	*	*	38	*	*	110	112	*
Triticum aestivum	536	109	*	326	*	*	364	71	*
Ambrosia artemisiifolia	877	253	270	456	365	12	151	*	*
Cirsium arvense	48	293	350	52	164	483	89	179	76
Polygonum pennsylvanicum	14	99	349	10	222	349	*	*	*
Setaria faberii	*	376	309	*	275	173	*	29	42
Chenopodium album	56	296	108	5	*	21	6	*	*

*Species with NPP values less than 5 $g \cdot m^{-2} \cdot yr^{-1}$

All values represent annual net primary productivity ($g \cdot m^{-2} \cdot yr^{-1}$)

TABLE 2

SUMMARY OF PLANT SPECIES COMPOSITION AND NPP CHANGES IN THE 4-YEAR, 5-YEAR, AND 6-YEAR OLD-FIELDS (1978-1980)

SPECIES	FERTILIZER			SLUDGE			CONTROL		
	4-yr	5-yr	6-yr	4-yr	5-yr	6-yr	4-yr	5-yr	6-yr
Festuca elatior	350	354	301	177	247	135	125	128	246
Poa pratensis	120	66	18	100	77	85	75	119	172
Solidago canadensis	120	448	270	112	569	865	21	142	159
Ambrosia artemisiifolia	*	169	397	*	*	227	*	*	*
Barbarea vulgaris	*	8	161	*	12	234	*	*	*
Setaria faberii	11	390	415	13	137	262	*	*	*
Oenothera biennis	*	14	76	*	*	62	*	*	*

*Species with NPP values less than 5 $g \cdot m^{-2} \cdot yr^{-1}$

All values represent annual net primary productivity ($g \cdot m^{-2} \cdot yr^{-1}$)

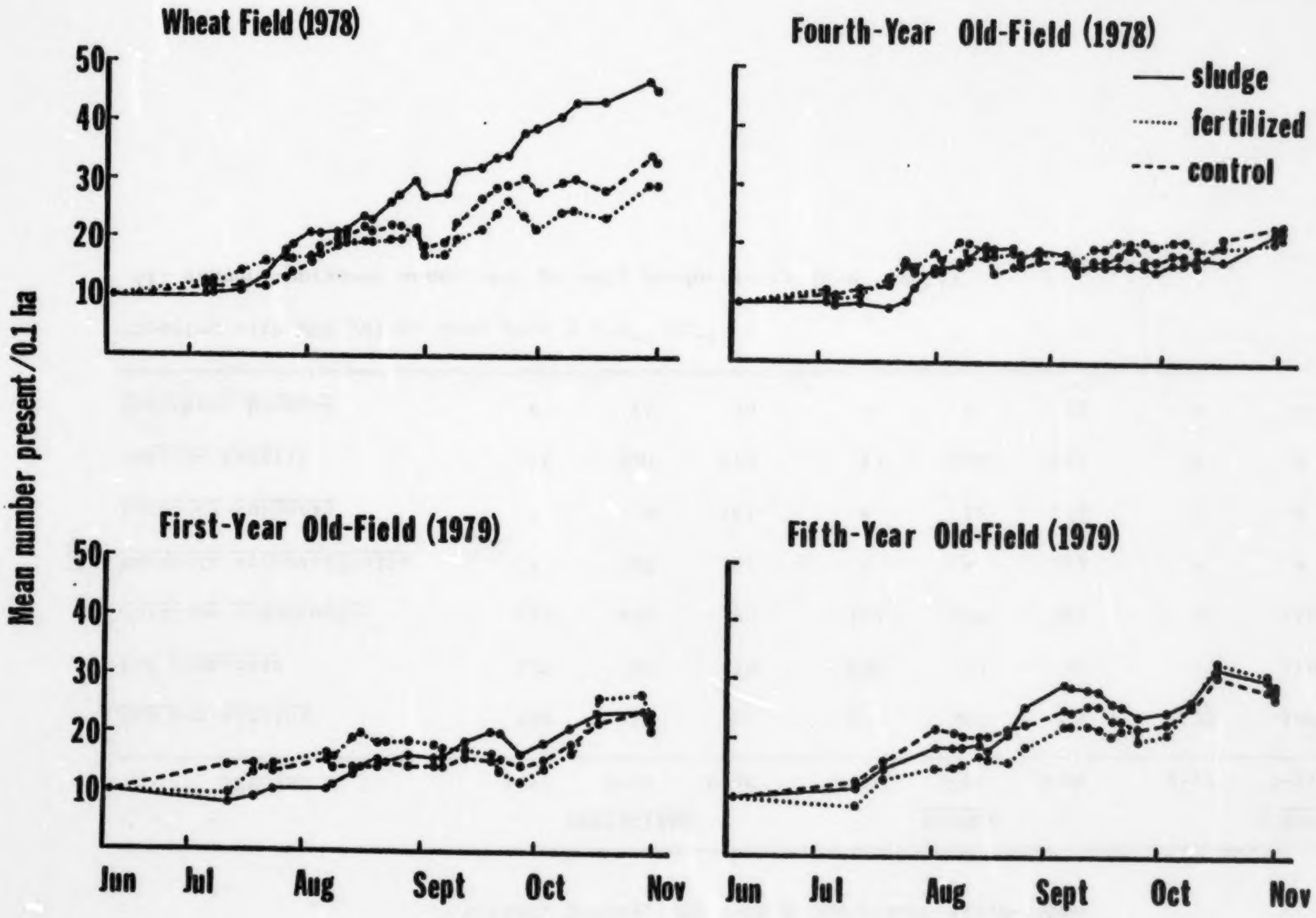

Figure 4. Mean meadow vole population densities per 0.1 ha.

ratios favored females in nutrient-enriched plots and males in control plots in 1978 and 1979; adult sex ratios did not differ significantly among treatments in either community-type in 1980. Recruitment rates (number of recruits/lactating female), however, were higher in control plots than in sludge or fertilizer enclosures during September and October in 1980. Also, fertilizer-treated plots had significantly lower population growth rates than sludge or control plots within the six-year old-fields.

METAL CONCENTRATIONS IN MEADOW VOLE TISSUES

Food-chain uptake of cadmium (Cd), lead (Pb), zinc (Zn), and copper (cu) by meadow voles was assessed in both community-types from 1978-1980; these metals will also be assessed in 1981. Thus far, only the 1978-1979 data have been completely analyzed.

Table 3 provides a summary of metal concentrations in Milorganite and commercial urea-phosphate fertilizer for 1978-1979. The 1980 concentrations, presently being analyzed, are comparable. There were no significant differences ($P > 0.05$) between years for cadmium, lead, or copper concentrations in either the fertilizer or the sludge. Zinc was significantly lower in the second year sludge samples compared to the first year. Cadmium was approximately 30 times higher in the sludge than in the fertilizer; lead 45 times higher; zinc 55 times higher; and copper 200 times higher.

Mean Cd concentrations in livers and kidneys of all sludge-treated groups in the second year of application (1979) were significantly higher ($P \leq 0.05$) than controls; in the first year (1978), only females from sludge-treated wheat plots had higher levels than controls. Tables of cadmium concentrations in vole tissue for both community-types are summarized in Tables 4 and 5. Remaining heavy metal data present in vole tissues for copper, lead, and zinc have been summarized by Anderson and Barrett (21). Ranges of mean Cd concentrations in sludge-treated voles were 0.1-1.1 μg/g wet weight in liver samples and 0.4-6.6 μg/g in kidneys; ranges in controls were 0.01-0.4 μg/g in liver and 0.01-1.6 μg/g in kidneys. Cd concentrations in fertilizer-treated voles did not differ from controls. These data have been submitted for publication (21).

Liver and kidney Cu concentrations were elevated in some sludge and fertilizer-treated groups. There were no significant differences in lead or zinc concentrations, nor in organ or whole-body weights.

Despite accumulation of metals in vital organs, short-term effects on small mammals due to sludge application appear to be benign, i.e., detected amounts are less than those volumes reported for laboratory feeding studies (22, 23).

METAL CONCENTRATIONS IN SOIL AND EARTHWORMS

Earthworms are a good indicator species for investigating heavy metal accumulation (24). They also play an important role in maintaining aeration, water permeability, and mineral turnover of the soil. Approximately 90% of the worms collected in this study were Lumbricus rubellus. Earthworms can

TABLE 3

METAL CONCENTRATIONS IN MILORGANITE AND COMMERCIAL UREA-PHOSPHATE FERTILIZER. VALUES REPRESENT 95% CONFIDENCE INTERVALS BASED ON THREE SAMPLES PER APPLICATION DATE (i.e., n = 15 FOR EACH INTERVAL)

	µg/g			
	Cd	Pb	Zn	Cu
Milorganite				
1978	58.4-59.6	463.6-482.4	1043.9-1118.1	312.8-327.2
1979	56.1-59.9	443.1-474.9	922.3-1013.7	315.7-342.3
Fertilizer				
1978	1.8-2.2	1.2-9.6	19.3-24.3	1.6-2.2
1979	1.2-2.0	0-25.0	12.6-20.2	1.2-2.0

TABLE 4

CADMIUM CONCENTRATIONS[1] IN VOLES FROM THE WHEAT FIELD (1978) AND FIRST-YEAR OLD-FIELD (1979)

	Control		Fertilized		Sludge	
	1978	1979	1978	1979	1978	1979
Liver						
adult males	0.01 (3) e	0.03 (3) e	0.03 (5) e	0.04 (3) e	0.14 (4) cde	0.28 (4) cd
adult females	0.04 (4) e	0.06 (2) de	0.09 (5) de	0.09 (4) de	0.56 (5) b	0.96 (4) a
sub-adult males	0.01 (4) e	0.02 (3) e	0.03 (6) e	0.05 (5) e	0.20 (5) cde	0.47 (4) b
sub-adult females	0.02 (3) e	0.04 (2) e	0.04 (4) e	0.04 (3) e	0.29 (6) c	0.83 (4) a
Kidneys						
adult males	0.01 (1) f	0.16 (2) f	0.09 (3) f	0.31 (2) f	0.90 (3) def	1.72 (3) bcd
adult females	0.20 (2) f	0.59 (2) ef	0.31 (3) f	0.88 (3) def	2.16 (3) bc	3.59 (3) a
sub-adult males	0.01 (2) f	0.12 (2) f	0.06 (3) f	0.10 (3) f	0.63 (2) ef	1.71 (3) bcd
sub-adult females	0.05 (2) f	0.14 (1) f	0.04 (2) f	0.17 (3) f	1.37 (3) cde	2.34 (3) b
Lungs						
adult males	0.04 (2) ab	0.01 (2) b	0.02 (3) b	0.01 (2) b	0.01 (3) b	0.02 (2) b
adult females	0.02 (2) b	0.01 (1) b	0.01 (3) b	0.01 (2) b	0.03 (3) b	0.02 (2) b
sub-adult males	0.02 (2) b	0.01 (1) b	0.01 (3) b	0.01 (2) b	0.02 (2) b	0.01 (2) b
sub-adult females	0.04 (2) ab	0.01 (1) b	0.01 (2) b	0.01 (2) b	0.02 (3) b	0.07 (1) a
Gonads						
adult males	0.01 (2) a	0.01 (3) a	0.01 (4) a	0.01 (3) a	0.01 (4) a	0.01 (4) a

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[1] Means of treatment replicates (μg/g wet weight); the number of samples is in parentheses; means within each organ followed by the same letter are not significantly different ($p > 0.05$) (Duncan's multiple range test).

TABLE 5

CADMIUM CONCENTRATIONS[1] IN VOLES FROM THE FOURTH-YEAR (1978) AND FIFTH-YEAR (1979) OLD-FIELDS

	Control		Fertilized		Sludge	
	1978	1979	1978	1979	1978	1979
Liver						
adult males	0.02 (4) d	0.04 (2) cd	0.03 (7) d	0.04 (4) cd	0.17 (5) cd	0.27 (4) bc
adult females	0.26 (1) bcd	0.44 (3) b	0.11 (6) cd	0.12 (4) cd	0.45 (5) b	1.06 (4) a
sub-adult males	0.11 (3) cd	0.02 (3) d	0.01 (4) d	0.06 (5) cd	0.07 (4) cd	0.44 (4) b
sub-adult females	0.14 (4) cd	0.04 (3) cd	0.05 (4) cd	0.07 (4) cd	0.23 (4) bcd	0.40 (4) b
Kidneys						
adult males	0.15 (2) e	0.40 (2) de	0.14 (3) e	0.44 (3) de	0.81 (3) cde	1.80 (3) bcd
adult females	-	1.61 (2) bcde	0.59 (3) cde	1.30 (3) bcde	2.27 (3) b	6.57 (3) a
sub-adult males	0.15 (2) e	0.01 (2) e	0.34 (2) de	0.33 (3) e	0.39 (2) de	1.93 (3) bc
sub-adult females	0.70 (2) cde	0.18 (2) e	0.12 (2) e	0.55 (2) cde	1.10 (2) bcde	2.24 (3) b
Lungs						
adult males	0.01 (2) b	0.01 (2) b	0.01 (2) b	0.01 (3) b	0.03 (3) b	0.01 (2) b
adult females	-	0.02 (2) b	0.01 (3) b	0.01 (3) b	0.02 (3) b	0.02 (3) b
sub-adult males	0.01 (1) b	0.01 (1) b	0.01 (2) b	0.01 (2) b	0.01 (2) b	0.02 (2) b
sub-adult females	0.01 (2) b	0.01 (1) b	0.01 (2) b	0.01 (2) b	0.14 (2) a	0.02 (2) b
Gonads						
adult males	0.01 (3) a	0.02 (2) a	0.01 (4) a	0.01 (2) a	0.01 (4) a	0.01 (2) a

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[1] Means of treatment replicates (μg/g wet weight); the number of samples is in parentheses; means within each organ followed by the same letter are not significantly different ($p > 0.05$) (Duncan's multiple range test).

considerably influence the trace element content of a soil by redistribution and bioaccumulation. Bioaccumulation of heavy metals by earthworms has been found to occur naturally in unpolluted soils and the accumulation is relative to the concentration found in that soil (24). Cadmium, nickel and zinc have been found to be accumulated in concentrations greater than those present in the soil. This accumulation is significant when there are large amounts of metal being introduced into the soil. A study was begun in May, 1981, to investigate the earthworm population and its accumulation of heavy metals in sludge- and fertilizer-treated plots.

Two control, two fertilized and two sludge-treated plots were randomly selected from the three-year field. Five 10 cm^3 samples were taken from each plot for each sample date. Samples were hand sorted and the worms placed in petri dishes on moist filter paper for four days to void their guts of soil. Soil samples were oven-dried at 80° C for 24 hours and screened through a 1 mm sieve. Soil and worms were digested with nitric acid and aqua regia to remove organic material. Atomic absorption spectrophotometry was used to quantify concentrations of metals.

A total of four sample dates will be treated in this manner. Early results indicate a significant difference in biomass between the sludge-treated and the control or fertilized plots. Biomass collected in the sludge plots was almost twice that found in the fertilized or control plots (Table 6). Their high density may be an important factor in the conditioning of the sludge-treated soil.

Data for concentrations of cadmium, lead, and copper are presented in Table 7 (data for zinc are not available at this time). Copper and lead show very little accumulation in earthworms collected from the sludge-treated plots. However, cadmium is concentrated by a factor greater than 10. The possibility of biomagnification through the food chain to birds or small mammal consumers awaits further investigation.

BEHAVIORAL ANALYSIS

Urinary marking patterns were used to establish a dominance index for Microtus pennsylvanicus. Scrotal males, weighing 25 g or more, were placed in 46 x 46 cm boxes with a wire mesh bottom. Filter paper was used to obtain the marking patterns. Animals were left in the marking boxes for a 12-hour period without food or water. The filter papers were analyzed with an ultraviolet light and an acetate overlay consisting of 36 squares.

A dominance index was calculated from the equation proposed by Noyes et al. (25), where the index value is equal to the sum of the relative density, relative area, and relative frequency of urine marks. The index values ranged from 0.74 to 40.09. Plotting cumulative percent vs the dominance index values, a breakpoint in the curve of 16 was determined. Animals with an index value greater than 16 were considered to be dominant. Those animals that had an index value less than 16 were said to be subordinate. These social rank predictions were supported by random pairing tests in which predicted dominants won 15 or 16 matches. The establishment of a dominance index allows for a

TABLE 6

BIOMASS (GRAM WET WEIGHT) OF EARTHWORMS COLLECTED FROM FERTILIZED, SLUDGE AND CONTROL PLOTS

	SLUDGE	FERTILIZER	CONTROL
SAMPLE I 26 May 1981	22.25	10.31	11.28
SAMPLE II 16 JUNE 1981	20.42	9.26	10.59
SAMPLE III 25 JUNE 1981	10.75	6.97	4.10

TABLE 7

SOIL AND EARTHWORM CONCENTRATIONS (PPM; µG/G) OF CADMIUM, COPPER AND LEAD AND CONCENTRATION FACTORS; ESTIMATED ACCORACY ± 0.1

	SOIL	EARTHWORMS	CF*
Cd			
Sludge	1.8	24.6	13.67
Fertilizer	1.0	3.7	3.70
Control	0.4	1.3	3.25
Cu			
Sludge	15.7	2.9	0.18
Fertilizer	9.1	1.4	0.15
Control	8.9	0.1	0.12
Pb			
Sludge	37.0	2.9	0.08
Fertilizer	25.4	2.0	0.08
Control	23.4	1.6	0.07

*CF = Concentration Factor; amount present in earthworm divided by amount present in soil.

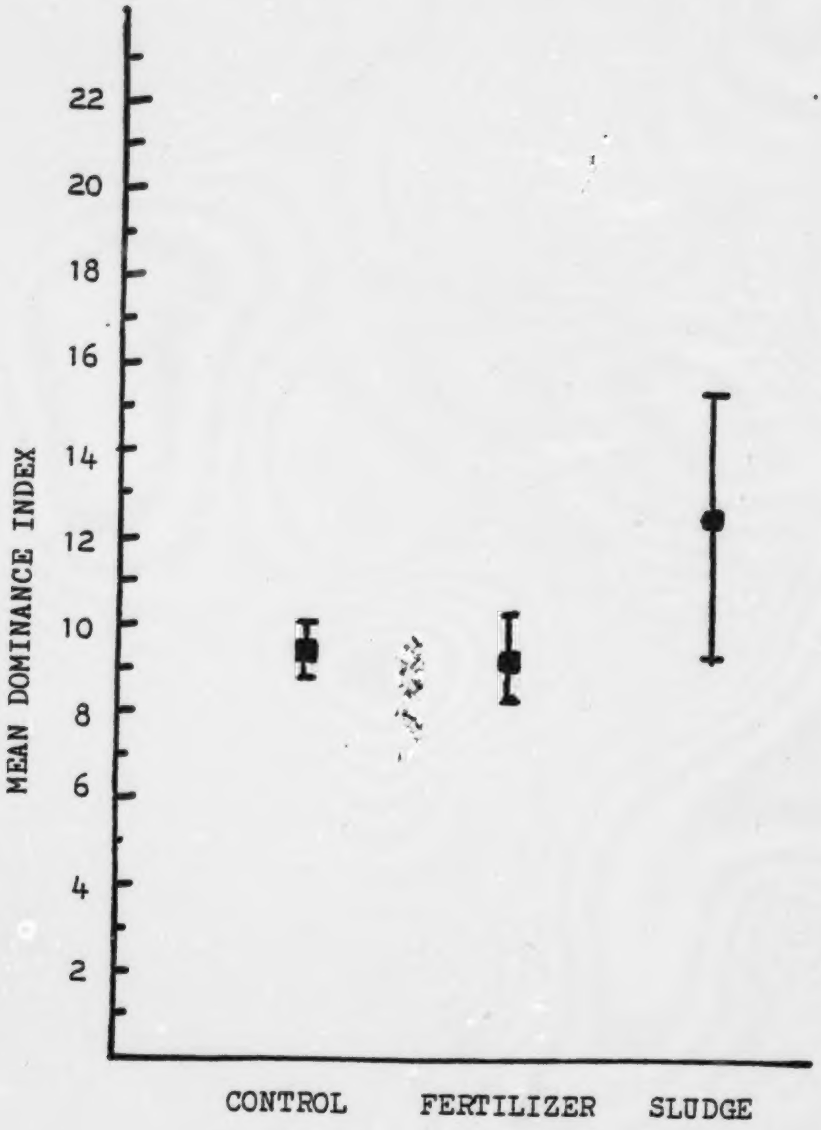

Figure 5. Comparison of the mean dominance index values and standard errors for *Microtus* males in each field treatment.

relatively quick method of constructing a social hierarchy in these microtine populations.

Figure 5 illustrates the mean dominance index values for Microtus populations in each of the experimental fields. The mean value in the control field is 9.80. The fertilized field shows a mean of 9.64 while the sludge-treated fields show a mean of 12.74. This difference between sludge and control plots is not significant but does suggest a possible trend.

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Comments on
Effects of Milorganite
on Two Grassland Systems

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In an unusual experiment, the Environmental Protection Agency has put its omniscient reviewers to the test: they have been asked to examine the preliminary results of an on-going research project which they themselves found worthy of support one or two years ago. What Barrett proposed at that time was:

a. to measure and evaluate the effects of chronic sludge application on several different levels of biological organization;

b. to compare two different grassland communities (types) treated in an identical manner;

c. to analyze different structural (e.g., biomass and species diversity) and functional (e.g., energy flow and community resilience) ecosystem parameters - measurements which may be indicative of total systems repsonse;

d. to evaluate sludge effects (via ecosystem food chains) on a natural small population (Microtus pennsylvanicus) functioning within these community types (we feel that wild mammal populations illustrate better the potential sludge effects on man than by extrapolating strictly from laboratory populations); and

e. to develop a feasible ecosystem approach, including the identification of "white mice" field parameters, for testing stressors (e.g., sludge) on intact ecosystems.

Individual aspects of these objectives had been examined earlier but no studies which attempted to do all this simultaneously had been undertaken. In effect, we were seeing a holistic approach and, therefore, a most ambitious one. As Barrett noted, Odum has observed that problems are created when stressors are tested at the individual level and then used without further study at the ecosystem level.

In addition to the prior stated goals anent total systems response and effects of sludge application on several different levels of biological organization, the PI hoped that the natural animal population, specifically Microtus pennsylvanicus, would serve better to illustrate the potential sludge effects on man than merely extrapolating from laboratory feeding experiments. In my own notes at the panel review I find that I raised the question as to whether Milorganite was equal to all sludges; implicit in this question was the idea that somehow Milorganite was better, cleaner (the psychological effect on me of commerical packaging, no doubt). A second question was whether the voles would breed at a differential rate sufficient to detect treatment effects on reproduction and behavior, as well.

The test plots were a 0.8 ha old-field (perennial) grassland and a 0.8 ha wheat field in its first year of old-field succession. Comparisons were made among 0.1 ha subunits enclosed with sheet metal and treated with Milorganite or commercial fertilizers or left as untreated controls and seeded with winter wheat or a mixture of grasses. No differences were observed in the first year between annual or perennial fields in species richness. Both commercial fertilizer and Milorganite increase productivity, the former earlier and to a greater extent. In the next two years, net primary productivity (NPP) was greater in one-year old-field fertilized and sludge plots as compared with control plots. This advantage was lost in two-year old-field studies; and in five-year and six-year old-fields only nutrient-enriched plots were greater in NPP than control plots. In contrast, control plots showed significantly greater species richness than nutrient-enriched plots in one-year and two-year old-fields. The differences disappeared in five-year or six-year old-fields.

At the primary consumer trophic level, Barrett found vole populations higher only in sludge-treated wheat plots. Although some significant differences in sex ratios were occasionally seen and recruitment rates appeared higher in control than treated plots. These data appear too preliminary to be taken seriously yet, as do lessened growth rates in fertilizer-treated plots in six-year old-fields. What is striking at this early stage of the study are the metal concentrations found in meadow vole tissues. It should be noted that Cadmium (Cd) was 30 times higher in Milorganite than in fertilizer, lead 45 times higher, zinc 55 times higher, copper 200 times higher. Analyses show Cd ranges in Milorganite-exposed voles to be 0.1 - 1.1 μg/g wet wt in liver, 0.4 - 1.6 μg/g in kidneys. Controls, by contrast, had 0.01 - 0.4 μg/g in liver and 0.01 - 1.6 μg/g in kidneys. In a preliminary experiment, Barrett compared Lumbricus rubellus harvested from control with those fertilized or sludge-treated plots and found, not unexpectedly, a mean concentration of Cd 10 times greater in the Milorganite-treated soils, the implications for biomagnification through birds and small animal predation needs further investigation.

I have hesitated, being normally of a pacific nature, to comment on the effects of sludge on dominance patterns. Although Barrett's data hint at increased dominance as shown by urinary marking patterns, I frankly don't know how to interpret the complex interactions of Milorganite spreading, elevated cadmium in livers and kidneys and urinary frequency, volume, and pattern. In the Philadelphia area, suburbanites in whose parks we attempt to spread composted sludge seem to react in similar fashion even before we dump the stuff.

I have done Dr. Barret somewhat of a disservice by summarizing his paper in unsophisticated fashion. What I wanted to note, however, is that there appear to be certain agricultural benefits to the use of some sludges. Concomitantly, we must recognize that not all sludges are created equal. Earthworms and vole kidneys make it apparent that heavy metal contributions from roads and parking lots as well as industrial processes are a significant problem. Nightsoils in Canton, while rich in pathogenic bacteria and parasites, don't have that particular problem. There are fields in the Braunschweig district in Germany which use liquid sludges in batch lots only after testing for the presence of excessive levels of heavy metals. Point sources can be controlled; street and road pollution cannot be in our older cities, in particular where common storm and sanitary sewer systems create a near unsuperable problem. For these cities, a return to selective ocean dumping, composting and subsequent application to mine spoils and parkways may yet be a more desirable choice.

I'd like to close with my favorite quotation from William Lowrance's book Of Acceptable Risk, as in a sense, I have highlighted some of Barrett's findings which have negative connotations. Lowrance wrote

> "In 1900 the principal insecticide, sprayed on everything from apples and grapes to strawberries and potatoes, was "Paris green" - lead arsenate. The first canned foods were being preserved with sulfites, boric acid, and formaldehyde at rather high concentrations. The leading antiseptic for home and hospital use was corrosive carbolic acid. The red robustness of teas, candies, and other commercial food products was imparted by lead chromate, which today's biochemist would prefer not even to handle, much less feed to anybody. Medicinals were unreliable: "Adulteration threatened the healing art. Even more alarming was the spread of proprietary or patent medicines. Some had genuine merit, but most were nostrums, often containing dangerous, habit-forming ingredients. Such fraudulent remedies were far from new, but in the latter part of the 19th century they increased in number and resort to consumption cures, lost-manhood tonics, soothing syrups, liver pills, and other so-called remedies became a craze." Foods were often adulterated; the laws controlling food composition were weak. Tinctures of opium such as laudanum and paregoric were readily available in

corner drugstores and were unchecked for indiscriminate use. Children frequently became habituated.

As to the general environment, some rivers were so filthy with raw sewage and industrial waste that, as the saying went, "bait died on the hook." Industrial towns were black with coal soot, as were people's lungs. Workers labored at their own peril."

"Since the taking of both personal and societal risks is inherent in human activity, there can be no hope of reducing all risks to zero. Rather, as when steering any course, we must continually adjust our heading so as to enjoy the greatest benefit at the lowest risk and cost."

THE ASSESSMENT OF TOXIC EFFECTS IN EXPERIMENTAL STREAM ECOSYSTEMS

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ABSTRACT

Fate and effects of toxic stress on large scale outdoor stream channels were studied from 1979 to 1981 at the Monticello Ecological Research Station, Monticello, Minnesota. P-cresol, an energy-related phenolic compound, was introduced at 8 ppm into closed-cycle stream ecosystems from 13 hours in 1979, for 24 and then 48 hours in 1980, and for 96 hours in 1981. This report presents only results from work in 1980. In 1980, microbiology and microcosm laboratories were added to the on-site facility to relate results from flask, microcosm, and pseudo-natural stream ecosystems to one another. The fate of p-cresol in microcosm tanks tracked that of the outdoor streams, but results from flask experiments did not track the fate of p-cresol in microcosm or stream ecosystems. The effects of p-cresol on the biological community during the 48-hour dose differed in each tier: flask, microcosm and outdoor stream channels. During the second night of the 48-hour dose in the stream channel, the concentration of dissolved oxygen sank to less than 1 ppm. By daybreak, several fish species were severely stressed as were amphipods, isopods and some species of mayflies. Neither severe oxygen depletion nor biotic stress occurred in microcosm or flask experiments. Changes in microcosm design were therefore made for the 1981 field season. Chemical and engineering advances were made in the on-line rapid detection of phenols. Sampling and behavioral methods were developed for the testing of effects of toxic compounds on natural biological systems.

INTRODUCTION

In the fall of 1978, Michigan State University was awarded a contract by the U.S. Environmental Protection Agency to design and implement a modification involving five of the eight 500 meter artificial stream channels at the Monticello Ecological Research Station, Monticello, MN., to study the fate and effects of toxic stress on large scale experimental stream ecosystems. The fundamental objective of the project has been, from the outset, to characterize the functional relationship between laboratory studies of toxicity and pseudo-natural ecosystem responses; i.e., to better define the rules of transferability and predictive robustness of flask, aquaria, and microcosm studies.

The results of that interdisciplinary design project are two independent, isolated, recycling stream ecosystems that physically, chemically, and biologically are typical of warm water, grassland, third-to-fifth order streams. Containing a water volume of 1.5 to 1.8 million liters each, the 1000 x 3 meters channels are driven by recirculation pumps that propel the stream at up to 11000 l/m resulting in mean velocities up to 12.2 cm/sec (riffle velocities range up to 25 cm/sec). The earthen channels have alternating riffles and pools of 30m lengths. The control and monitoring of the channel flows and water chemistry (pH, DO, temperature), local meteorological conditions, and toxicant injection are executed by a dedicated on-site computer system.

An organic chemistry laboratory was established on-site geared to our prototype toxicant, p-cresol. Thus, we have established, at the Monticello site, a set of large experimental stream ecosystems, and facilities for analyzing toxicant concentrations in all ecosystem components and microbial activities.

The work supported by the EPA Office of Research and Development involved the basic systems refinement, operation, and analysis for a 24-hour and a 48-hour ecosystem exposure to the toxicant, p-cresol, during the 1980 experimental season.

EXPERIMENTAL OPERATIONS

24-HOUR EXPOSURE

P-cresol was added at 8 ppm from 1330 hours on July 19th to 1300 hours on July 20th (Figure 1). The retention time for the channels was approximately 6 hours at a flow rate of 4000 liters per minute. By 2200 hours an equilibrium profile was established with a slight oscillation of concentration rippling through the system.

Figure 2 presents the ambient concentrations at the tail end of the system. Both the flow-through phenol analyzer and the gas chromatographic analyses produced similar estimates. The estimated half-life was approximately 64 hours for the first 24 hours during the dosing. After dosing terminated, accidental events and a misjudgement of the process of p-cresol degradation prevented us from monitoring until the following morning. At 1100 hours we picked up the analysis at 2 ppm ambient levels and measured half-lives less than one hour.

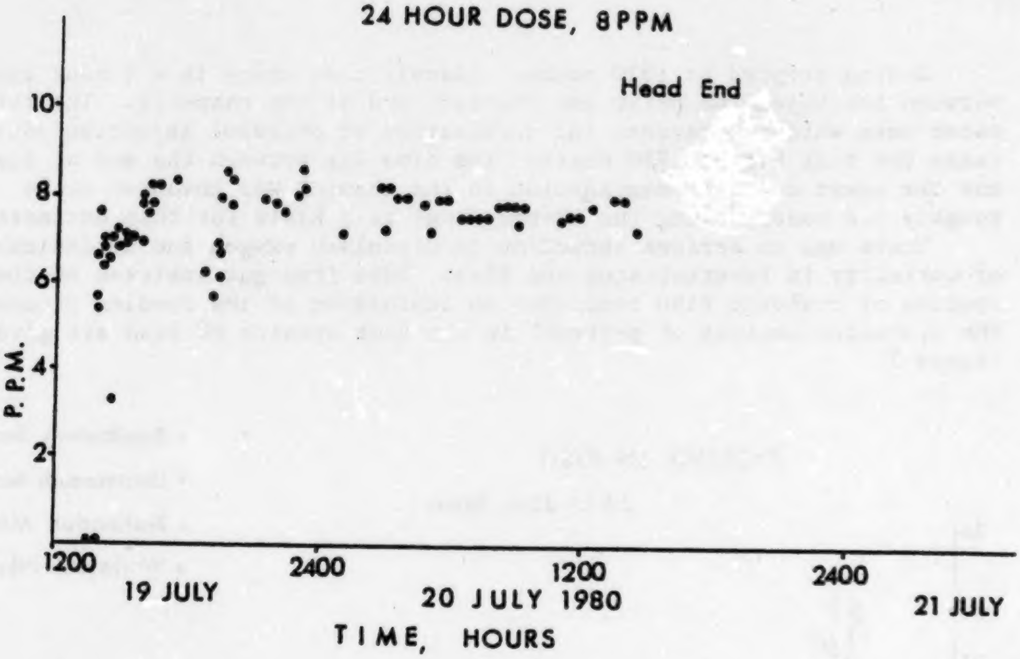

Figure 1. Ambient Concentrations of p-Cresol at Head End of Channel B During and After Dose of p-Cresol at 8 ppm. July 19-21, 1980.

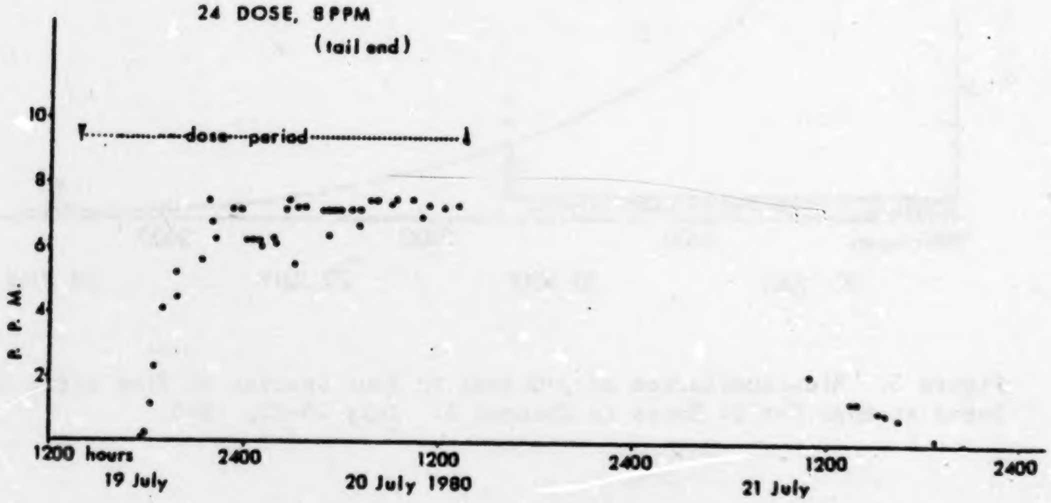

Figure 2. Chemical Analyses of a 24-hour Dose of p-Cresol at 8 ppm in Channel B at the Tail End of the Channel. July 19, 20, 1980.

Dosing stopped at 1330 hours. (Recall that there is a 6 hour time lag between the injection point and the tail end of the channel). The first water mass which represents the termination of p-cresol injection would reach the tail end at 1930 hours. The time lag between the end of dosing and the onset of rapid degradation in the channel was computed to be roughly 3.5 hours, using the 48-hour dose as a basis for this estimate.

There was no serious reduction in dissolved oxygen and no indication of mortality in invertebrates and fish. Data from gut analyses of the four species of resident fish indicated an inhibition of the feeding process. The bio-accumulations of p-cresol in the four species of fish are given in Figure 3.

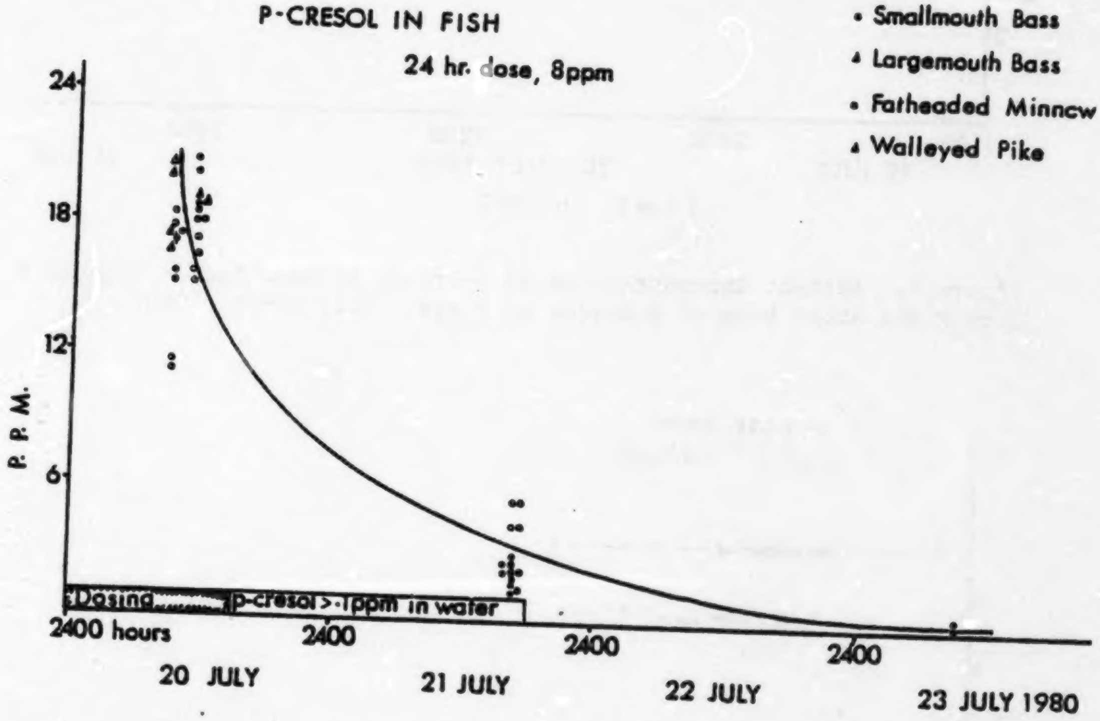

Figure 3. Bioaccumulation of p-Cresol in Four Species of Fish After Being Dosed at 8ppm for 24 Hours in Channel B. July 20-23, 1980.

The average total body load was 18 ppm, which represents a bioaccumulation factor of 2.3. The average water ambient levels varied from 7.5 to 8.0 ppm until approximately 2300 hours on July 20. All four species showed the same accumulation and release pattern. By 1100 hours on July 21, the ambient concentrations were reduced to 1 ppm and the fish contained between 2 - 5 ppm; 24 hours later only trace amounts were measurable in the fish;

they had a very rapid release of p-cresol. Bacterial samples demonstrated a change in microbial flora associated with the p-cresol application. The number, size, and color of bacterial plaques shifted dramatically during the 24 hour period of injection. Initial attempts to demonstrate significant increases in p-cresol degradation by these new bacteria were not definitive. Figure 4 presents the bacterial density estimates. The four sampling stations in Channel B were distributed uniformly along the stream. The dosing period was from 1330 hours, July 19, to 1330 hours, July 20. Bacterial counts increased consistently in Channel B but no increase was observed in the control stream. The increased density was maintained for at least five days after dosing ceased, and concentration of p-cresol was 0.5 ppm by July 21 (Figure 2).

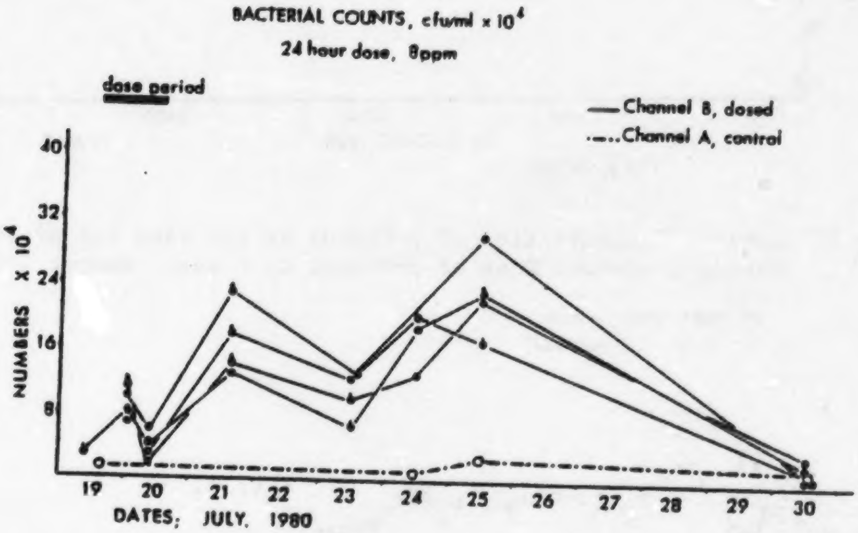

Figure 4. Bacterial Density Counts (CFU/ml x 10^4) in Channel A (Control) and Channel B (Experimental) During and After a 24-Hour Dose of p-Cresol at 8 ppm in Channel B.

48-HOUR EXPOSURE

The 48 hour dose occurred between 1000 hours, August 9, to 1000 hours, August 11. Ambient concentrations were maintained around 8 ppm (Figure 5). Again, the p-cresol appeared to be very stable during the 6 hour retention time in the recyling mode.

Figure 6 presents the ambient concentration of the tail end of the stream just before the water entered the sump to be recycled. The 6.25 hour retention time is illustrated by the fact that dosing started at 1000 hours and the first wave of p-cresol reached the last sampling station at approximately 1530 hours. The oscillations were produced by periodic problems with the dosing apparatus. By comparing Figures 5 and 6, an estimated average of 1.7 ppm phenol was lost during the 6.25 hour retention time. This would produce an estimated 14.7 hour half life, assuming an exponential drop in concentration over the stream.

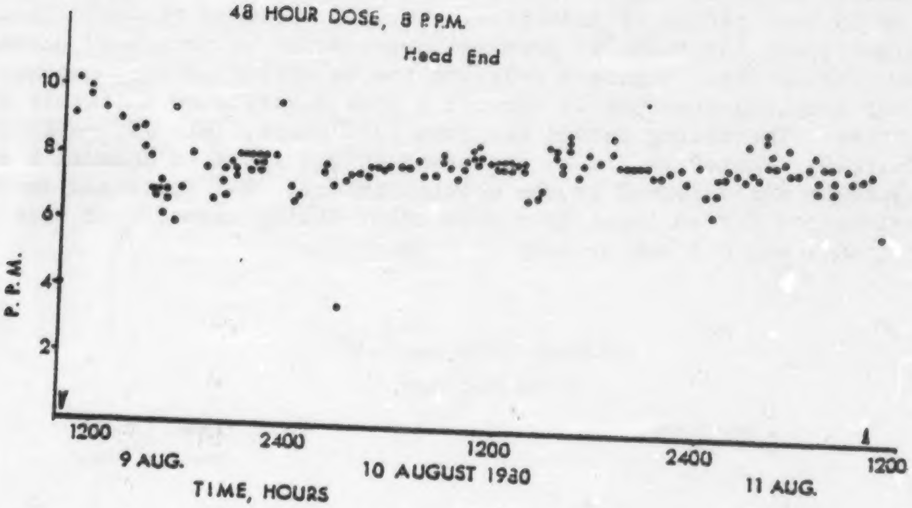

Figure 5. Ambient Concentration of p-Cresol at the Head End of Channel B During a 48-Hour Dose of p-Cresol at 8 ppm. August 9-12. 1980.

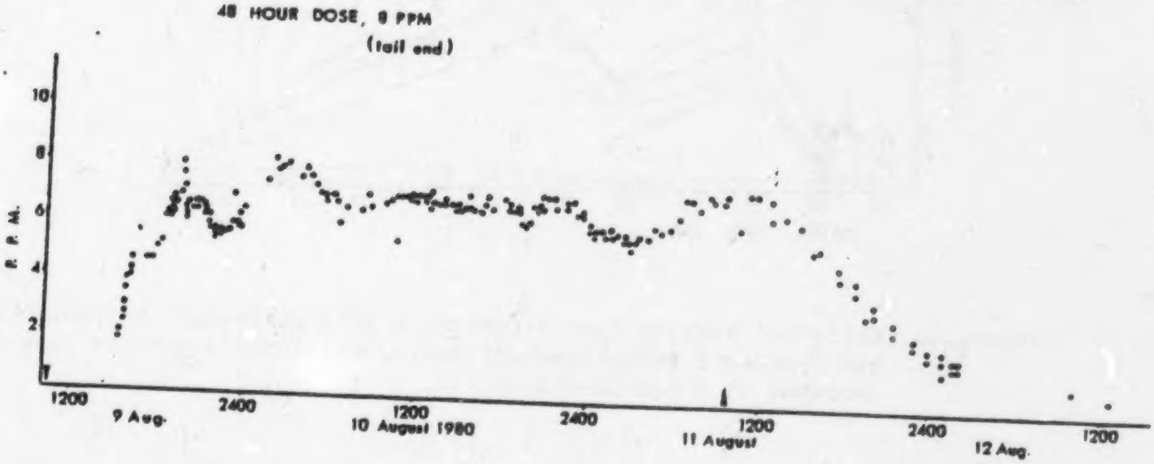

Figure 6. Chemical Analyses of a 48-Hour Dose of p-Cresol at 8 ppm in Channel B. (Tail End of Channel). August 9 - 11, 1980.

The inflection point should not have occurred before 1615 on August 11 since the dosing stopped at 1000 and the retention time was 6.25 hours. There appeared to be no time lag in degradation response, as the ambient concentrations declined immediately. The compared half-life during the decrease from 6 ppm to 2 ppm was approximately 5.3 hours. Obviously, the degradation rate increased significantly after the dosing terminated.

Examination of the differences in total phenol measurements between tail end of the stream and head end of the stream indicate that between hour 14 and hour 30 of the 48 hour dosing, the differences steadily increased, from an interpolated half-life of 69 hours to a half life of 23 hours. Also at hour 31 a decrease in concentration at the tail end of the stream (when the head end remained relatively constant) implies 2 further decrease in half life from 23 hours to 12 hours. This is much closer to the half life immediately after dosing (5.3 hours). Also, immediately before dosing ended, a difference between tail end and head end of 2.5 ppm was apparent, which would give a half life of 11.1 hours. Thus, adaptation by microbial populations to faster biodegradation rates was probably occurring in the stream. During the dosing experiments, the temperature showed normal diurnal variation from 22 to 27°C, and the pH varied from 7.0 to 8.5. However, dissolved oxygen showed a marked change during and after dosing which was obviously related to the effect of p-cresol as shown in Figure 7. The D.O. changes triggered a number of secondary biological effects

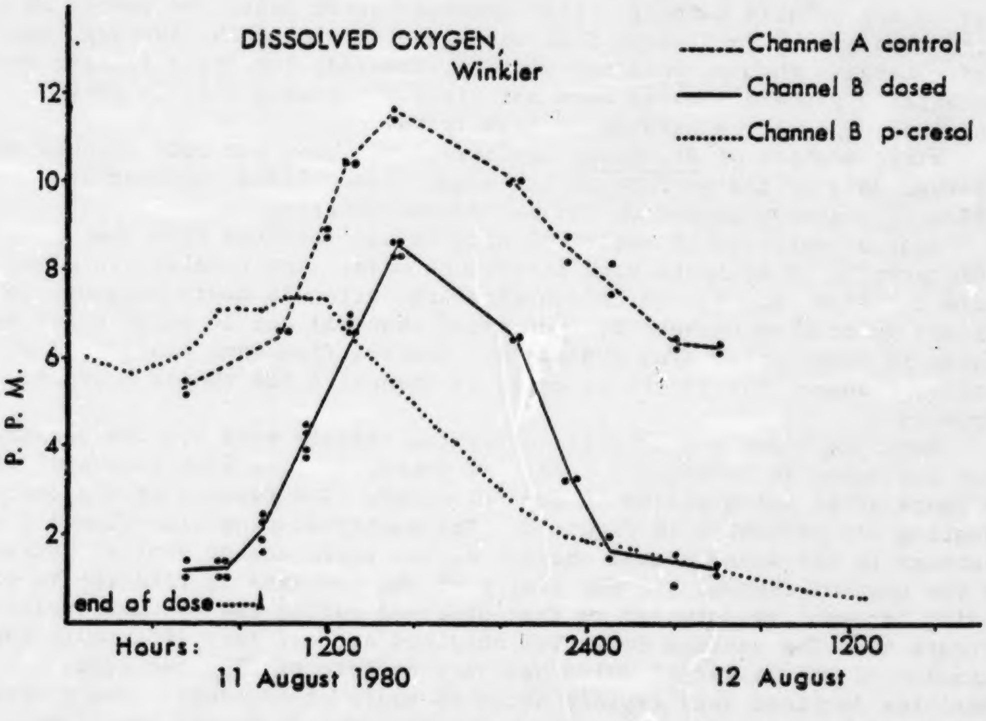

Figure 7. Dissolved Oxygen Profiles in Channel A (control) and Channel B (experimental) with p-Cresol Levels (48 hour dose).

The dissolved oxygen profile of the control stream demonstrated the normal diurnal cyclicity between 4 and 12 ppm.
It is hypothesized that the mechanism of effect is probably the increased respiration of organisms (plants, animals and microbes) and not the inhibition of photosynthesis. The dissolved oxygen curves for the control and dosed streams are approximately parallel, with the major difference being the minimal levels reached at 0500 to 0600 hours; i.e., the rate of photosynthesis was similar and the pattern of the diurnal cycle was not disturbed. Chemical oxidation of p-cresol is possible, yet the dissolved oxygen increased during daylight at the same rate as the control stream and the ambient levels of phenols were 7 to 8 ppm. The dissolved oxygen was reduced to low levels of 1 to 1.5 ppm during the night even though the phenol levels dropped to less than 2 ppm, suggesting that nighttime D.O. sags can be either intensified at low p-cresol concentrations, or the higher concentration of p-cresol the previous day had a lag effect, which was expressable at night.

During the periods of low dissolved oxygen on both August 11 and 12, certain fish and invertebrates experienced massive mortality. Walleye pike died in appreciable numbers. Fish removed before death and placed in clean water recovered. Smallmouth bass were gasping air at the surface, but none died. Largemouth bass were not visibly stressed, but their feeding was inhibited. Fathead minnows were not visibly stressed and, in fact, deposited eggs on the surface of fish traps.

Large numbers of Stenonema mayflies, amphipods and some isopods were observed dead on the surface of the algal mats. Field observations indicated a severe impact on the macroinvertebrates.

Another estimate of nonlethal effects was obtained from the predator-prey experiments with largemouth bass. The results are given in Table I. Fish were tested before exposure, after 36 hours exposure to ambient water from Channel B, (the dosed channel) and 24 hours after being placed in clean water from Channel A. Control fish were handled in a similar fashion, but placed in cages in Channel A for the 36 hours of exposure.

Handling times and percent successful capture were the two parameters that indicated an inhibitory effect of p-cresol. The fish recovered within 24 hours after being placed in control water. The results of the bacterial sampling are presented in Figure 8. The bacterial densities showed a sharp increase in the experimental channel B; but there was no similar increase in the control Channel A. The timing of the increase in relation to the dosing interval was similar to that observed during the 24-hour application (Figure 4). The maximum densities obtained are not very different, but the duration of the bacterial pulse was very different. The bacterial densities declined very rapidly after 48 hours of exposure. The p-cresol concentration declined much more slowly than the bacterial densities.

Table I. Predation Parameters for Largemouth Bass Exposed to p-Cresol

	P-cresol Exposed Fish			Control Fish	
	Before	During	Recovery	Before	During
Encounter Rates (per sec.)	.037	.0152	.034	.045	.028
Mean Search Times (sec.)	31.15±32.6	40.4±69.5	33.52±35.0	28.2±28.4	39.88±41.9
Handling Times (sec.)	8.94±3.34	18.25±11.4	9.6±5.0	8.32±3.97	7.00±2.6
% Capture	100%	47%	94%	97%	97%

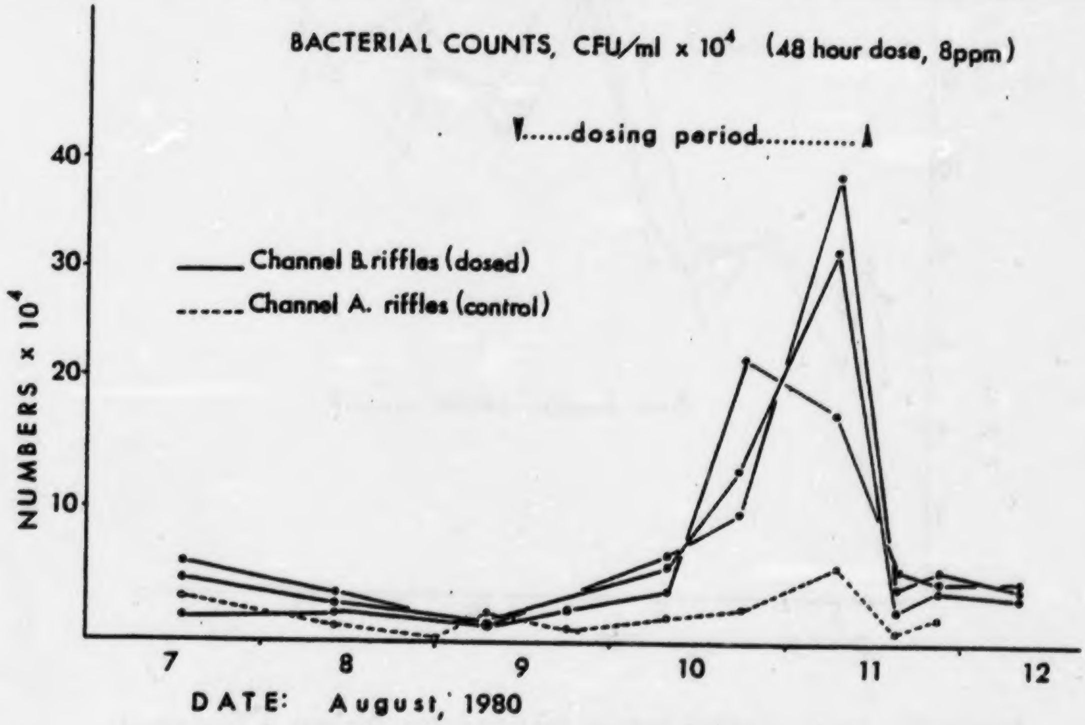

Figure 8. Bacterial Counts (CFU/ml x10^4) in Channel A (control) and Channel B (Experimental) Before, During and After 48-Hour Dose of p-Cresol in Channel B. August 7-12, 1980.

Total organic carbon (TOC) analyses are presented in Figure 9. The baseline levels were between 3.5 and 8.0 ppm in both channels. The stations sampled were distributed uniformly along

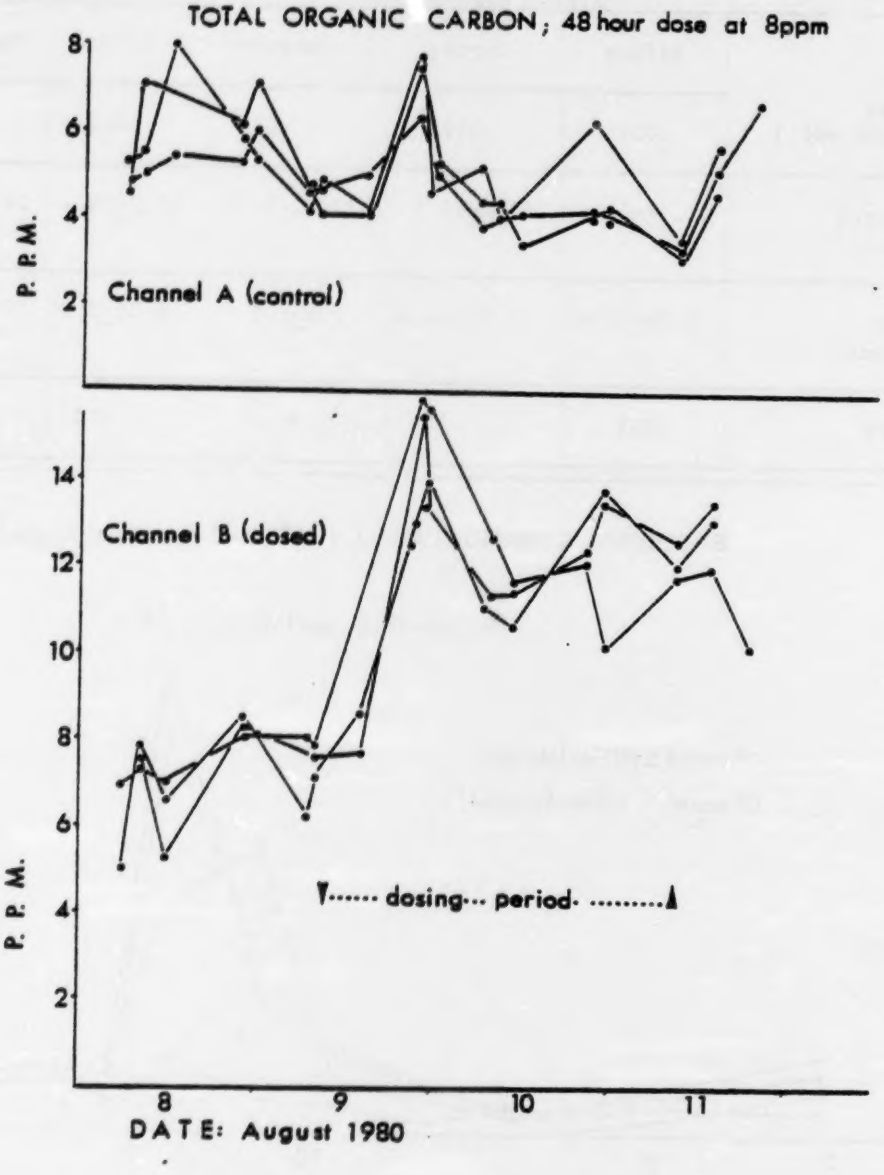

Figure 9. Total Organic Carbon Analysis for Channel A (control) and Channel B (experimental) Before and During Dosing of Channel B with p-Cresol at 8 pp for 48 hours. August 8 - 11, 1980.

the streams. The sharp increase in TOC observed in the dosed channel is probably due to two sources. P-cresol is 77% carbon by weight. P-cresol was added to maintain 8 ppm which produces 6 ppm additional organic carbon. The average carbon for Channel B before dosing was about 7 ppm. The additional carbon associated with p-cresol would raise the ambient TOC level to 13 ppm. The observed levels peaked at 16 ppm on the night of the initiation of dosing. P-cresol also will increase the respiration rates of some organisms due to stress. Additional evidence of the potential impact of increased respiration is seen in Figure 7. The reduction in dissolved oxygen observed at 0600 hours on August 11 must have occurred during the previous two nocturnal periods. The differences in dissolved oxygen levels is about 4 ppm on August 11.

Another source of TOC would be the population increase of bacteria (Figure 8); however, the timing of the TOC and bacterial population curves are not synchronized. The TOC indicated peak values during the first 24 hours of dosing; whereas, the bacterial peak occurred during the second 24 hours. Furthermore, the dissolved oxygen data do not indicate that the stress factor, i.e., increased respiration in response to p-cresol, had diminished by August 11.

ANALYSES

BIOACCUMULATION

Table II gives the results of the bioaccumulation of p-cresol by five species of fish that were sampled during and after the 48-hour dose. Twenty-four hours into the 48-hour dose, walleye pike and largemouth bass showed similar levels of p-cresol to those found near the end of the 24-hour dose. P-cresol concentrations for those species and for bluegills and fatheads remained high throughout the dosing. Twenty-six hours after dosing stopped, p-cresol levels in the fish dropped. The fish accumulate the toxicant rapidly, but they also release rapidly when ambient levels of p-cresol are low.

Bluegills collected 20 hours after cessation of the 48-hour dose were dissected for organ analyses. The eye (without lenses) had the lowest concentration (2.8 ppm) and muscle tissue the next lowest (4.7 ppm). Gill tissue contained 7.3 ppm. The highest bioaccumulation values were in the livers (76.1 ppm) and in the intestines (96.7 ppm). High accumulations in the liver suggests sequestering of p-cresol from blood tissue. The very high values in the intestines could possibly occur if the fish began eating soon after dosing ceased. The results from fish gut analyses show that bluegills stopped feeding during dosing but began feeding within hours after dosing stopped. Even so, the high accumulation in the intestines was unexpected. Skin was not analyzed but it is expected that the accumulation probably was low, given the fact that whole bluegills taken four hours later only contained 12 ppm p-cresol.

Various biotic components of the channel were analyzed for p-cresol cocentration. Table III shows that only one component of those tested, namely a crayfish, showed significant amounts of p-cresol. The crayfish, *Orconectes virilis*, bioaccumulated nearly three times more p-cresol than background concentrations in the channel. This crustacean is the largest

TABLE II

BIOACCUMULATION* OF P-CRESOL IN FISH DURING AND AFTER 48 HOURS OF DOSING CHANNEL B AT * PPM P-CRESOL

		P.P.M. OF P-CRESOL IN FIVE SPECIES OF FISH				
DATE	**TIME**	**Walleye Pike**	**Largemouth Bass**	**Bluegill Sunfish**	**Fathead Minnow**	**Smallmouth Bass**
8/10	1000		18.7	5.9		
			12.0	13.7		
8/10	1300	17.5	18.6		12.4	
		18.9	16.8		17.8	
		14.4	16.7			
			13.4			
8/11	0530			14.9		
8/11	0745	15.4				
		14.8				
8/11	1000		15.9	24.5		
				29.0		
8/11	2330			14.0		
8/12	1000		20.3	12.0		
8/12	1200		4.8		0.9	
8/15	2000					0.5

*Gas Chromatograph Data

TABLE III

CONCENTRATION OF P-CRESOL IN SEDIMENTS, PLANTS AND INVERTEBRATES AFTER 48 HOURS OF DOSING CHANNEL B AT * PPM P-CRESOL

		P.P.M. OF P-CRESOL IN COMPONENTS			
DATE	**TIME**	**Sediment**	**Algae Spirogyra**	**Dragonfly Anax**	**Crayfish Orconectes**
8/10	1000		0.8		
			0.0		
8/10	1100	0.9			
8/11	0945			0.8	23.9
8/11	1000	3.8	1.1		
		1.8	4.0		
8/12	1000	0.7			
		0.0			
8/14	1000		0.0		

and is one of the most common invertebrates in the channel. It is a detritus feeder but also can capture other invertebrates and slow-moving fish; they possibly could have consumed stressed fish during dosing, which could account for the high concentrations of p-cresol in the crayfish.

TOXICITY

Selected fish and invertebrates from the sites were tested at the E.P.A. Laboratory at Duluth for laboratory response to p-cresol. Those data appear in Table IV. The fish species show a distinct "all-or-none' pattern: fatheads, for the most part, died after 48 hours in 20 ppm p-cresol, but none died (even after 96 hours) with concentrations of 10 ppm. Largemouth bass all died after 24 hours at 32 ppm, and none died at 16 ppm; smallmouth bass died after 24 hours at concentrations of 20 ppm, but all survived at 10 ppm. The damselfly, Ischnura verticalis showed no response, even at concentrations of 40 ppm. The amphipod, Hyalella azteca showed a more gradual dose-response pattern. For short periods of time (24 hours) most of the animals survived, even at concentrations of 80 ppm, but only 50% survived after 72 hours at concentrations of 10 ppm. Death also occurred at concentrations of 5 ppm after 48 hours. The waterflea, Daphnia magna, was very sensitive to the toxicant at 2.5 ppm, even after 24 hours. During our 48-hour dose, we noted dead amphipods caught on algae.

BEHAVIORAL RESPONSES

Preliminary data show that there was a volumetric increase in invertebrates in drift nets during dosing of p-cresol. We also observed qualitative increases in numbers of animals during dosing. The preliminary data bear this out. At the end of the summer, we varied velocity of Channel B (500, 1000 and 2000 gal/min) and took drift samples during each velocity change. There was more than a two-fold increase in drift between 1000 and 2000 gal/min, but there was little difference between 500 and 1000 gal/min.

TABLE IV

DOSE-RESPONSE DATA ON P-CRESOL WITH SELECTED FISH AND INVERTEBRATES FROM MONTICELLO, MINN.

Mount and Norberg
E.P.A. Lab. at Duluth

Organism (number)	Initial Concentration of P-Cresol	TIME			
		24-hour % Survival	48-hour % Survival	72-hour % Survival	96-hour % Survival
Fathead	40 mg/l	60, 30	20, 0	0, 0	0, 0
minnow	20 mg/l	60, 90	30, 40	0, 10	0, 0
(10/run)	10 mg/l	100,000	100,000	100,000	100,000
	5 mg/l	100,000	100,000	100,000	100,000
2 replicates	2.4 mg/l	100,000	100,000	100,000	100,000
	Control	100,000	100,000	100,000	100,000
Largemouth	32 mg/l	0	-	-	-
bass	16 mg/l	100	100	100	100
(5/run)	8 mg/l	100	100	100	100
	4 mg/l	100	100	100	100
1 replicate	2 mg/l	100	100	100	100
	Control	100	100	100	100
Small mouth	40 mg/l	0	-	-	-
bass	20 mg/l	0	-	-	-
(5/run)	10 mg/l	100	100	80	80
	5 mg/l	100	100	100	100
1 replicate	2.5 mg/l	100	100	100	100
	Control	100	-	-	-
Largemouth	20 mg/l	0	-	-	-
bass	10 mg/l	100	100	100	100
Test #2	5 mg/l	100	100	100	100
(5/run)	2.5 mg/l	100	100	100	100
1 replicate	1.25 mg/l	100	100	100	100
	Control	100	100	100	100
Damselfly	40 mg/l	-	100		
(10/run)	20 mg/l	-	100		
	10 mg/l	-	100		
1 replicate	5 mg/l	-	100		
	2.5 mg/l	-	80		
	Control	-	70		

TABLE IV, continued

Organism (number)	Initial Concentration of P-Cresol	TIME 24-hour % Survival	48-hour % Survival	72-hour % Survival	96-hour % Survival
Amphipod	80 mg/l	50,37.5	0, 0	0, 0	0, 0
Hyalella	40 mg/l	87.5,50	25, 25	12.5,12.5	12.5,12.5
azteca	20 mg/l	87.5,100	37.5,50	37.5,37.5	12.5, 25
(8/run)	10 mg/l	100,000	75, 87.5	50, 50	27.5, 50
2 replicates	5 mg/l	100,000	87.5,87.5	87.5, 50	75, 37.5
	Control	100,000	100,000	100,000	100,000
Waterflea	40 mg/l	0, 0	0, 0		
Daphnia	20 mg/l	0, 0	0, 0		
magna	10 mg/l	0, 0	0, 0		
(5/run)	5 mg/l	60, 80	60, 40		
2 replicates	2.5 mg/l	80, 80	60, 20		
	Control	100,000	100,000		

1. Flow per minute in chambers ranged from 21 to 60 ml/min.
2. Initial D.O. values ranged from 5.5 to 7.9 ppm
3. Final D.O. values ranged from 5.5 to 8.4 ppm
4. pH ranged from 7.2 to 7.5
5. Alkalinity = 40; hardness = 46
6. Temperature was 24 to 26^{0}C.
7. Measured concentration of p-cresol was done for each run. Those data are not presented here, but are part of the Mount and Morberg report to us and are available on request.

Comments on
The Assessment of Toxic Effects in Experimental Stream Ecosystems

Robert J. Beyers
University of South Alabama

In order to gain a complete understanding of the effects of any toxicant on an ecological system, one must undertake studies on many different organisms at various trophic levels within that system. Removing organisms from their natural environment and challenging them with pollutants always carries the possibility of missing synergistic effects which may take place within the system. However, it is frequently undesirable from a practical or esthetic point of view to choose a natural system and deliberately dose it with a toxic substance. In order to obtain better control and to protect the environment from harm caused by the experiment, ecologists have frequently relied on some form of microcosm experiment when engaged in this type of investigation.

This is precisely the situation in the Boling-Cooper work. In this case they have chosen to use a large artificial stream facility located at the Monticello Field Station. These streams are large enough to be called "mesocosms" by the investigators. The advantage of large size is that reasonable amounts of biota can be removed during sampling without destroying the structure of the systems. Also larger organisms can complete their life cycles in a normal manner. A further advantage of this kind of experiment is that the inputs can be carefully controlled, in this case by a computer.

Finally, an artificial stream is basically a closed system without immigration or emigration from the outside. This is effective in simplifying the mathematical models which best describe the workings of a biological community. It may be assumed that deviation from the model of an unstressed community will be an indication of the reaction of an ecosystem undergoing toxicological stress.

For these reasons, the Environmental Protection Agency Environmental Review Panel felt that this work would be a prototype for the kinds of experiments which must be widely done in the future to determine the effects of various pollutants on total ecosystems, as opposed to studies on individual "indicator" organisms. In this light the proposal was considered highly significant, and funding was recommended.

The preliminary results of the present work bear out these contentions. The sheer size of the system permitted observations on the behavioral aspects of selected organisms and the analysis of bioaccumulation in certain members of the system.

It is to be hoped that in the future, additional work and analysis of these and new data will bear out the expectations of the Environmental Protection Agency. Some biomagnification was observed (algae vs. crayfish), but some surprises were also noted (low concentration of toxicant in predators such as small mouth bass).

POLYPHOSPHATE IN NATURAL PHYTOPLANKTON ASSEMBLAGES AND ITS FORMATION AND DEGRADATION IN CULTURED ALGAL CELLS

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INTRODUCTION

A survey of stations in Saginaw Bay of Lake Huron during the period 1974-1976 demonstrated that: 1.) Certain phytoplankton populations were exiting the bay and surviving transport to southern Lake Huron (27), 2.) Many of these populations in the bay were found to have polyphosphate accumulations in them (28), and 3.) Several of the populations of blue-greens and diatoms in the bay had lead sequestered in the polyphosphate bodies (22, 21). Moreover, it has been demonstrated with both cultured blue-greens (3, 1) and diatoms (22) that heavy metals may be incorporated into polyphosphate bodies during phosphate uptake.

Polyphosphate is believed to function primarily as a phosphorus reserve (19). It can be formed under several distinct nutritional conditions: 1.) Restoration of phosphate following a phosphorus deficiency (19, 14), 2.) Nutrient imbalance other than phosphorus (16, 29, 23, 24), and 3.) Disturbance of nucleic acid metabolism (11).

The eventual fate and ecological significance of polyphosphate is not well known. If polyphosphate serves as a phosphorus storage form that can be degraded under conditions of limiting phosphorus, then phosphorus limitation should make polyphosphate (and heavy metals if they are so bound) available to intracellular sites.

In view of the fact that Saginaw Bay is the only major basin in the Great Lakes that has experienced a substantial reduction in phosphorus loading during the last 5 years, samples were collected from Saginaw Bay to determine what effect, if any, this phosphorus loading reduction had on the distribution of polyphosphate in natural phytoplankton assemblages. In addition, laboratory experiments were conducted to determine both the time sequence and consequences of polyphosphate degradation in cultured algae as well as the effects of heavy metal exposure at the time of luxury phosphorus uptake. Preliminary results are presented in this paper.

MATERIALS AND METHODS

Field samples were collected in conjunction with the 1980 Saginaw Bay and southern Lake Huron Special Field Survey conducted under the auspices of the Great Lakes National Program Office and the Grosse Ile Large Lakes Research Laboratory of the U.S. E.P.A. Whole water samples were collected from Saginaw Bay approximately every two weeks from the period April 14, 1980 through November 14, 1980 and from southern Lake Huron during five cruises beginning in May 1980 and ending October 1980. Selected stations in the segment scheme of Saginaw Bay as well as a series of stations along the western shore of southern Lake Huron were sampled as weather permitted.

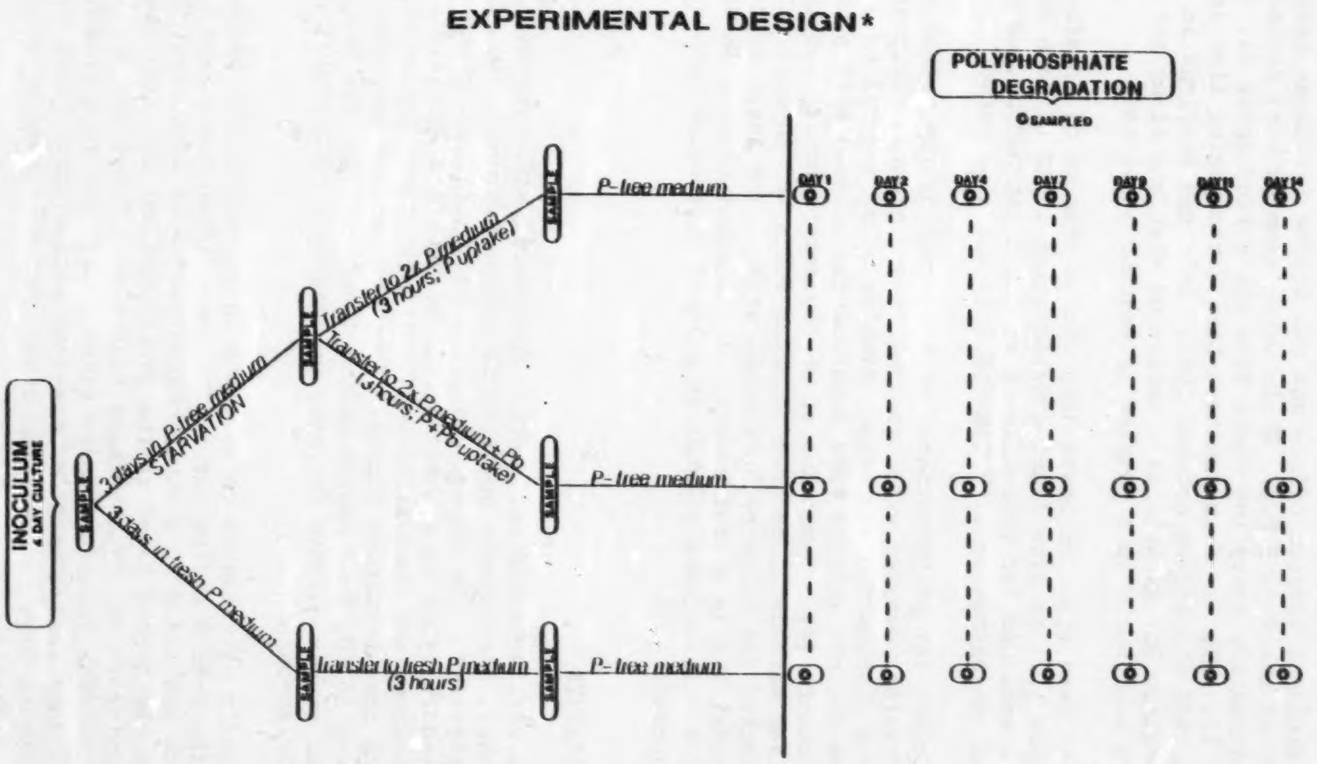
Water samples were fixed on board ship with a mixture of paraformaldehyde-glutaraldehyde (17) to give final concentration of 1% of each aldehyde. For samples to be embedded for transmission electron microscopy, cacodylate buffer at a final concentration of 0.05M (pH 7.2) was also added.

Samples examined for polyphosphates were stained by the method of Ebel et al. (7). Initially, samples were examined in wet mounts. However, due to paucity of certain species and vibration, samples were filtered onto 0.8 μm Millipore filters and the filters were subsequently cleared with glutaraldehyde for actual counts (6). Summaries of phytoplankton counts for the Saginaw Bay cruises were obtained from computer files and phytoplankton species were selected for polyphosphate enumeration. The three criteria for selection of species within a cruise were: 1.) Species present on 50% or more slides, 2.) Species always present at $\geq$ 1% of assemblage numbers, and 3.) Maximum occurrence $\geq$ 5%.

LABORATORY EXPERIMENTS

Three algae were selected for study: *Cyclotella* aff. *meneghiniana* (Bacillariophyceae), *Scenedesmus quadricauda* (Chlorophyceae), and *Plectonema boryanum* (Cyanophyceae). The algae were grown to logarithmic phase at 20°C on a 12/12 light-dark cycle in either WC (10) or modified Fitzgerald's medium (8). Both *Scenedesmus* and *Cyclotella* were initially grown separately in WC medium in Corning tissue culture flasks. However, for the experiment, beginning with transfer to PO_4-free medium before luxury uptake, the two were combined and treated as one culture for ease of handling. *Plectonema* was maintained separately throughout.

The algal cells were washed three times in phosphate-free medium prior to transfer to the P-free medium for starvation. After three days in P-free medium, phosphate was added to a final concentration of approximately 8 mg PO_4/liter (twice the normal level in the growth medium) and lead was added with thorough mixing in the Pb treatment flasks to a final concentration of 20 ppb. The cells were incubated under these conditions in a growth chamber for three hours, then washed in phosphate-free medium three times prior to transfer to phosphate-free medium. The cells were then sampled according to the schedule in Figure 1.



EXPERIMENTAL DESIGN*
POLYPHOSPHATE DEGRADATION
SAMPLED
INOCULUM
4 DAY CULTURE
SAMPLE
3 days in P-free medium
STARVATION
3 days in fresh P medium
SAMPLE
Transfer to 2x P medium
(3 hours; P uptake)
Transfer to 2x P medium + Pb
(3 hours; P+Pb uptake)
SAMPLE
Transfer to fresh P medium
(3 hours)
SAMPLE
SAMPLE
SAMPLE
SAMPLE
P-free medium
P-free medium
P-free medium
DAY 1
DAY 2
DAY 4
DAY 7
DAY 9
DAY 11
DAY 14

Figure 1. Experimental design of polyphosphate-lead uptake and degradation studies. This design was used for two separate cultures--a mixed culture of *Scenedesmus* and *Cyclotella*, and a unialgal culture of *Plectonema*. *Sampling was later extended to 3 weeks.

For every sample withdrawn as indicated, cells were treated in the following manner:

1. Unfixed cells were washed 3 times in sterile distilled water. Drops of cell suspension were then dried on formvar coated nylon grids for x-ray analysis.

2. Portions of cells fixed in paraformaldehyde-glutaraldehyde were withdrawn, stored in fixative, and subsequently stained for polyphosphates.

3. The remainder of the fixed sample was then post-fixed in 1% OsO_4, dehydrated in a graded ethanol-propylene oxide series and embedded in Epon for transmission electron microscopy.

For quantitative electron microscopy, random sections were obtained through embedded pellets of the algae. Thirty micrographs at 75,000 X were examined for each treatment by superimposing a transparent 1.0 cm square sampling lattice over the picture. Estimates of volume density were obtained using the grid point-counting technique (2, 9).

RESULTS

MORPHOLOGICAL CHANGES (*PLECTONEMA BORYANUM*)

Detailed ultrastructural examination of controls, phosphate starved, and phosphate uptake cells of *P. boryanum* have been published elsewhere (13). The present experiment differs from that report in regard to the length of phosphate starvation. Quantitative results of the experimental treatments are presented in Table 1. In general, phosphate starvation results in a decrease in the surface area to volume ratio (S_V) of thylakoids. This low ratio is also observed when either P + Pb uptake cells or logarithmic phase cells are again placed in phosphate-free medium. Polyhedral body relative volume (V_V) appears to increase slightly during P starvation. Polyphosphate body (V_V) increases during phosphate uptake and subsequently decreases during P starvation. The number per volume (N_V) increases substantially during uptake. During the uptake process, with or without lead, polyphosphate bodies are relatively small and scattered throughout the cell (Figures 2-3). However, fewer numbers are present during subsequent P deprivation and they are both larger and located in areas of medium electron density (Figures 4-5). Images of what we interpret as polyphosphate degradation are present as early as 2 days in the P deprivation medium. Polyphosphate bodies appear to be less electron dense; they are more prone to volatilization in the electron beam and often only a small dense portion is left at the periphery of the body.

Qualitative observations were also made of cells incubated for 4 and 7 days in P deprivation medium after phosphate uptake. Virtually all cells exposed to lead during phosphate uptake were dead at four days. The sample also contained cellular debris, indicating lysis. The P uptake cells as well

TABLE 1. MORPHOMETRIC RESULTS OF PHOSPHATE AND LEAD TREATMENTS. SURFACE AREA/VOLUME (S_V); RELATIVE VOLUME (V_V): NUMBER/VOLUME (N_V). RESULTS ARE THE MEAN ± 1 S.E.

	Treatments*								
	INOC	P STRV	P SUFF	P-UP	PB-UP	C-UP	P DEG2	PB DEG2	C DEG2
Thylakoid (S_v)	20.24 (1.14)	14.57 (0.93)	17.79 (1.35)	18.34 (1.29)	15.30 (1.20)	17.19 (1.15)	19.64 (1.45)	14.39 (1.34)	14.32 (0.93)
Poly P (N_v)	12.48 (5.78)	66.38 (17.82)	156.81 (33.63)	113.91 (12.66)	396.45 (53.87)	201.88 (38.36)	79.75 (15.40)	62.59 (12.90)	197.27 (30.64)
Poly P (V_v)	0.42 (0.10)	0.48 (0.15)	0.45 (0.11)	1.77 (0.19)	2.06 (0.33)	1.46 (0.30)	0.51 (0.11)	0.37 (.09)	1.25 (0.24)
Polyhedral (N_v)	2.48 (0.53)	4.37 (0.91)	2.83 (0.49)	2.71 (0.44)	2.26 (0.49)	2.01 (0.45)	2.18 (0.39)	3.81 (0.87)	4.17 (0.70)
Polyhedral (V_v)	2.00 (0.38)	3.17 (0.45)	2.30 (0.44)	2.21 (0.42)	1.53 (0.30)	1.50 (0.32)	2.44 (0.45)	1.91 (0.38)	2.44 (0.35)
Intrathylakoidal Space (V_v)	12.67 (1.26)	9.08 (1.24)	4.91 (0.70)	4.06 (0.74)	10.62 (1.29)	13.14 (1.01)	9.37 (1.07)	7.02 (0.84)	10.06 (1.13)
Other (V_v)	84.90 (1.26)	87.26 (1.34)	92.34 (0.73)	91.95 (0.81)	85.79 (1.36)	83.90 (1.11)	87.68 (1.13)	90.70 (0.88)	86.25 (1.33)

*Treatment Code:
INOC = Inoculum
P STRV = P starved 3 days
P SUFF = P sufficient 3 days
P-UP = P uptake 3 hours
PB-UP = P + PB uptake 3 hours
C-UP = New transfer P sufficient cells 3 hours
P DEG2 = P uptake--2 days in deprivation medium
PB DEG2 = P + PB uptake--2 days in deprivation medium
C DEG2 = P sufficient cells--2 days in deprivation medium

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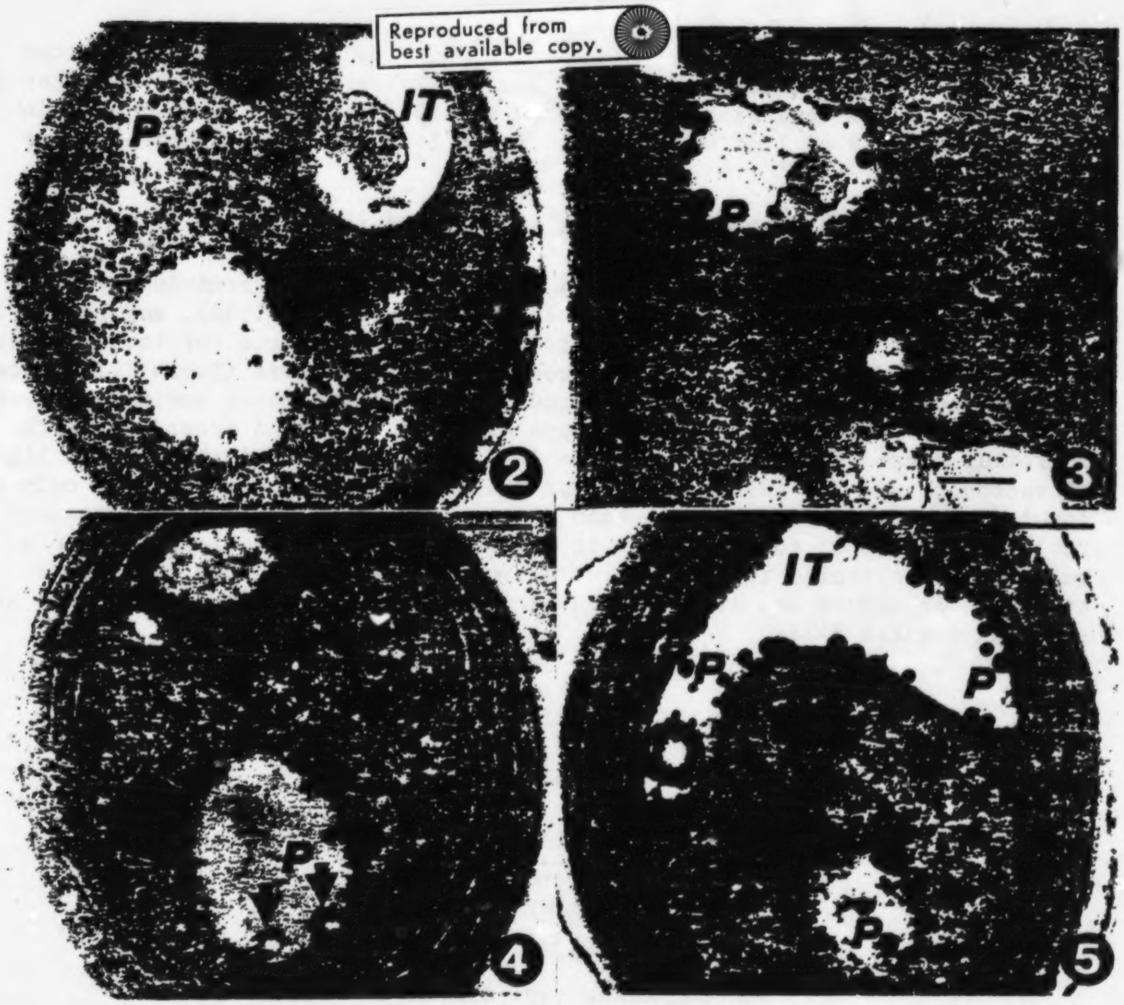

Figures 2-5. *Plectonema boryanum*. Magnification bars = 0.2 µm. Intrathylakoidal space (IT), Thylakoids (T), PolyP (P), Polyhedral bodies (PH).

Figure 2. Phosphate uptake cell (3 hours). Note light areas of intrathylakoidal space. Numerous polyphosphate bodies are scattered throughout the cell. Thylakoids are also evident.

Figure 3. Phosphate + lead uptake (3 hours). Greater numbers of polyphosphate bodies are present with this treatment.

Figure 4. P uptake cells incubated 2 days in P deprivation medium. Note fewer numbers of polyP and altered sturcture (arrows).

Figure 5. Day 2 deprivation medium of cells not previously incubated in starvation medium. PolyP bodies are larger and more numerous than in other P deprivation treatments.

as those maintained in medium containing phosphorus began to decline after four days in P-free medium. However, live cells were still observed after 7 days. When cells were examined by x-ray energy dispersive analysis, no detectable quantity of lead was observed in cells subjected to lead exposure during P uptake.

POLYPHOSPHATE DISTRIBUTION IN SAGINAW BAY CRUISES 1 AND 2

Species summaries were generated for the Saginaw Bay cruises where the phytoplankton counts were complete. For cruise 1 (April 1980), and cruise 2 (May 1980), 15 and 17 species respectively met the criteria for inclusion as key species. There were 13 species common to both cruises (Table 2). Since many of the species present had numerous small polyphosphate bodies that were difficult to quantitate, the cells were examined and rated according to the following scheme: 0 = no polyP, +1 = less than 5 bodies visible in the light microscope, and +2 = numerous (greater than 5). These numbers relate only to light microscopic examination. Each station for the 2 cruises was examined for the presence of polyphosphate in the key species. Polyphosphate (on a relative scale from 0 to 2) was plotted on maps of Saginaw Bay to compare trends in abundance and distribution with station. Several of these maps are shown in Figures 6-11.

TABLE 2. SPECIES COMMON TO BOTH CRUISES 1 AND 2 OF SAGINAW BAY POLYPHOSPHATE SURVEY. CRUISE 1. APRIL 14-18, 1980; CRUISE 2. MAY 5-8, 1980

Cyclotella comensis
Chroomonas spp.
Diatoma tenue var. *elongatum*
Dinobryon spp.
Flagellate spp.
Fragilaria capucina
Fragilaria crotonensis
Ochromonas spp.
Rhodomonas minuta
Scenedesmus spp.
Stephanodiscus subtilis
Stephanodiscus tenuis
Synedra filiformis

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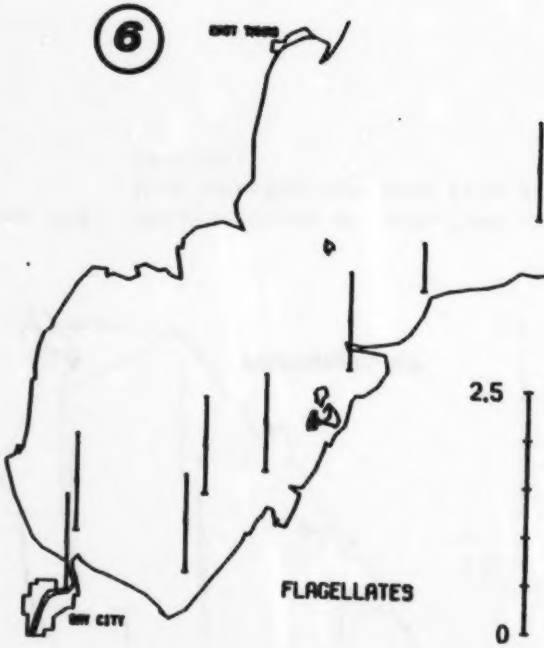

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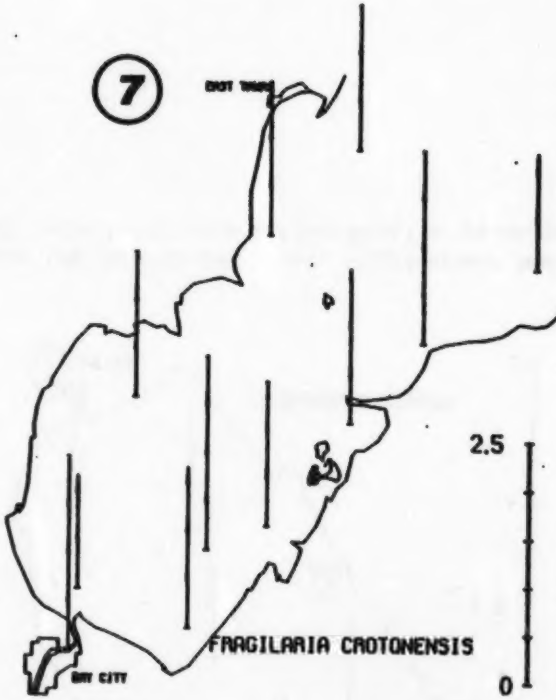
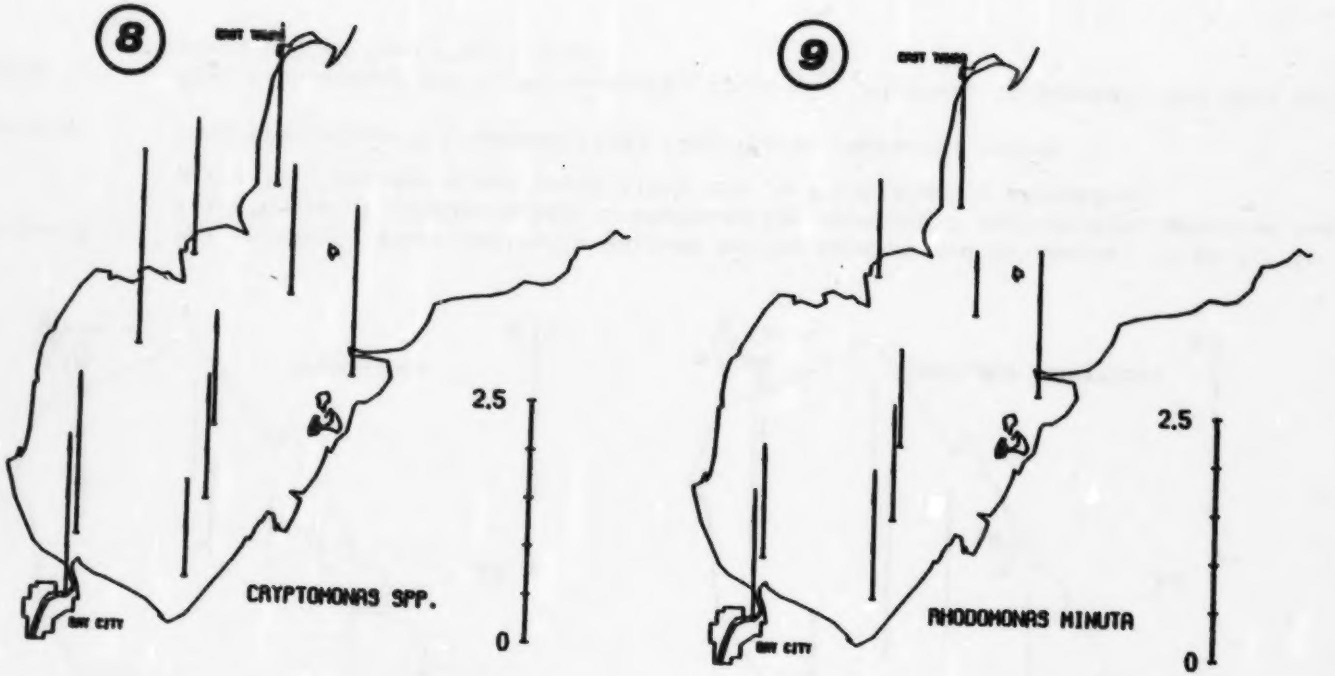


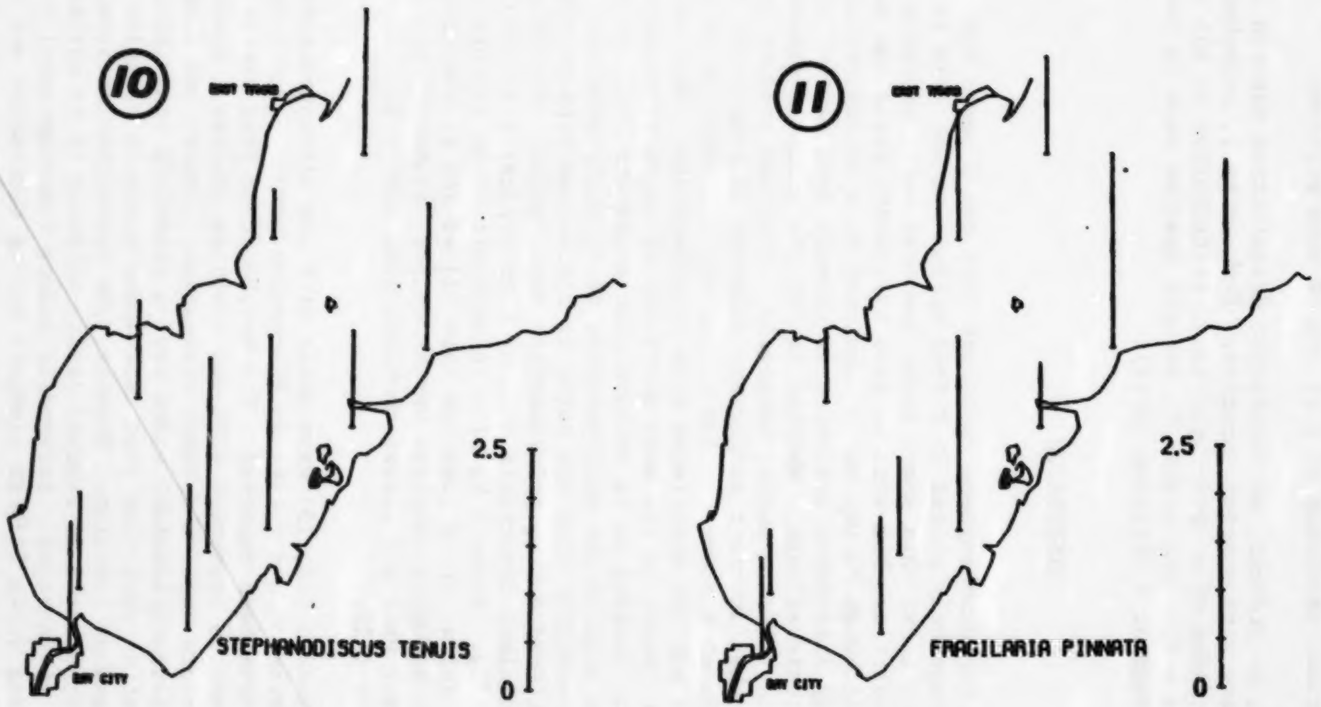
Figures 6-11. Polyphosphate distribution in Saginaw Bay by species and abundance. 0 to 2.5 is a relative scale of abundance with 0 representing none and 2 representing numerous bodies per cell. Abundance and scale lines are in a north-south direction.

Figure 6. Poly P abundance for unidentified flagellates category, cruise 2.

Figure 7. Poly P abundance for *F. crotonensis*, cruise 2. Diatoms, in general, had more polyphosphate bodies than flagellates.


8
EAST TAWAS
BAY CITY
CRYPTOMONAS SPP.
2.5
0
9
EAST TAWAS
BAY CITY
RHODOMONAS MINUTA
2.5
0

Figures 8-9. Polyphosphate distribution and abundance for two flagellates, *Cryptomonas* and *Rhodomonas*. Most species examined from cruise 1 had relatively equal distribution patterns throughout the bay.


⑩
EAST TAWAS
BAY CITY
STEPHANODISCUS TENUIS
2.5
0
⑪
EAST TAWAS
BAY CITY
FRAGILARIA PINNATA
2.5
0

Figures 10-11. Polyphosphate distribution and abundance for two diatom species from cruise 2. In the second cruise, relatively fewer bodies were counted in cells along the northern shore. This is the area of entry of open Lake Huron water.

Most flagellates with the exception of *Rhodomonas minuta* possessed far fewer polyphosphate bodies than either diatoms or greens (Figures 6-7). Early May samples had more polyphosphate across all species than did the April samples. For example, in cruise 1 (April) only 4 of 8 diatom species examined had a maximum abundance for all stations of 2, whereas in cruise 2, 9 of 10 diatoms had a maximum abundance of 2 at one or more stations.

There appears to be, at present, no consistent distribution pattern or diminution of polyphosphate with station location. For cruise 1, polyphosphate body abundance is random with apparently equal distribution in all segments of the bay (Figures 8-9). For cruise 2, several species seem to have fewer polyphosphates in segment 4 (Figures 10-11).

DISCUSSION

Cells of *Plectonema boryanum* exposed to 20 ppb lead for 3 hours during phosphate uptake and subsequently placed in P-free medium are not able to survive longer than 2 days after this short term, low dose metal exposure. Under similar culture conditions, but with no lead treatment, cells can survive a week or longer. Although Pb was not accumulated to a concentration detectable by x-ray energy dispersive analysis, it appears that this metal was capable of exerting toxic effects. Whether or not the metal is associated with polyphosphate is, at this point, uncertain. Previous studies in our lab (21) demonstrate that nutrient sufficient cultures of *Plectonema* exposed for seven days to Pb at a concentration of 0.1 μg-at/l resulted in lead accumulation in the cells but not associated with polyphosphate. This seven-day exposure also did not result in the mass mortality of cells experienced in the present experiment, leading us to believe that phosphate nutrient status may play an important role in the manifestation of toxicity symptoms. There still remains a possibility that the metal is associated with polyphosphate and that it is mobilized during polyphosphate degradation. Shuman et al. (18) found that the minimal detectable mass of K in typical biological thin sections was 10 mmol/kg. Since 1 kg of a logarithmic phase culture contains roughly 5.5×10^{12} cells, it is possible that [1] we are at the limits of detection at 20 ppb Pb assuming complete uptake and/or "trapping" and [2] Pb diffuses across the cell wall and plasma membrane more slowly in *Plectonema* than in *Diatoma* (22).

Although previous reports (13, 15) have dealt with the ultrastructural aspects of phosphate starvation and uptake in *Plectonema boryanum*, no quantitative cytological data have been reported. The morphometric data presented in this paper generally are in agreement with our previous studies. However, several cellular compartments deserve further discussion. Jensen and Sicko (13) noted increases in intrathylakoidal space during phosphate starvation and uptake. The quantitative data show that this space actually occupies a larger volume in cells used as inoculum. However, the vacuolization appears to be coalesced into a larger area in starved cells, allowing us to earlier interpret this change as an increase. Polyhedral bodies (carboxysomes) were also observed by Jensen and Sicko (13) to elongate during starvation and uptake. No elongated polyhedral bodies were observed in this study. The increase in relative volume (V_v) appears to be a direct result of an increase in number per volume.

Polyphosphate relative volume for inoculum, starved, and uptake cells correlates well with previous results (20) which demonstrated that logarithmic phase cells have low reserves of both acid soluble and acid insoluble polyphosphates. No changes in cyanophycin granules were observed in this study during phosphate starvation. Jensen and Sicko (13) reported an increase in these inclusions after 5 days starvation in *P. boryanum* and similar results have also been reported for *Agmenellum* (25).

The cells held in medium containing phosphorus also experienced a large increase in polyphosphate N_v with no concommitant increase in relative volume. Doonan (5) demonstrated that 14-day logarithmic phase cells produce alkaline phosphatase, which is not constitutive in *P. boryanum* nor is it released to the medium. Rapidly growing cells take up phosphate when transferred to new medium (19, 5). In the present experiment, this increase was solely an increase in the numbers of very small (slightly larger than ribosomal size) polyphosphate bodies.

Cells transferred to medium devoid of phosphorus after uptake responded differently, depending on the treatment history of the cells. Those cells which had been subjected to a period of phosphorus starvation experienced larger decreases in polyphosphate relative volume after 2 days in the deprivation medium.

The polyphosphate bodies formed during uptake change cytologically during phosphate deprivation. In addition to there being fewer bodies present, there appears to be a degradation sequence which resembles a reverse of the formation sequence described by Jensen (12) and Stewart and Alexander (26). We observed increased numbers of "porous structures" thought to be the site of polyphosphate deposition as well as images of polyphosphate bodies where there was only a remnant of electron dense polyphosphate at the periphery. As the length of time in deprivation medium increases, the frequency of observation of these forms also increases.

Previous survey observations (27) demonstrated that polyphosphate bodies were abundant in phytoplankton species that were distributed primarily along the southern and southwestern shore of Saginaw Bay. This study also showed that polyP was particularly abundant in blue-greens and was not found in species of the Cryptophyceae. Although we have only compiled data for the first two cruises, several features are already noteworthy. Polyphoshate has been found of several members of the Cryptophyceae and is also found in a variety of organisms along the northern shore, an area where previously none was found. There is no reason to believe, at this time, that polyphosphate abundance has declined as a result of a decreased phosphorus loading to the bay, although preliminary phytoplankton species composition and abundance data would indicate that shifts occurred in species composition and numbers of algae were reduced.

The results of the experimental uptake and degradation experiments suggest that if species containing polyphosphate are transported out of Saginaw Bay into southern Lake Huron, they would have a survival advantage unless metals were incorporated in polyphosphate. Two additional organisms, *Cyclotella meneghiniana* and *Scenedesmus quadricauda*, were also included in the experimental treatments described earlier. Data collection at this time is incomplete. However, light microscopic examination of *Scenedesmus* revealed that the cells survived longer than *Plectonema*, implicating more tolerance and/or exclusion of the metal. Samples have also been collected from the western shore of southern Lake Huron. The normal water circulation of the bay is counterclockwise with water exiting the bay along the southern shore and being replaced by water entering the bay along the northern coast (4). Stoermer and Kreis (27) have shown that populations which originate in Saginaw Bay (e.g. *Fragilaria capucina*) under certain conditions can survive transport into the extreme southern part of Lake Huron. Survey data is still being compiled from these two areas. However, we hope to determine if there is a reduction in polyphosphate as the algae are transported into the less nutrient rich waters of Lake Huron and whether there is any correlation with species abundance in the lake and polyphosphate numbers in Saginaw Bay.

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Comments on Polyphosphate in Natural Phytoplankton Assemblages and its Formation and Degradation in Cultured Algal Cells

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Dr. Sicko-Goad proposed a three-year field and laboratory investigation to: 1) determine the relationship of polyphosphate formation to dispersal and dominance of nuisance producing phytoplanktonic populations in aquatic systems, 2) determine if polyphosphate formation is a significant mechanism in uptake, biological transport, and bioaccumulation of toxic heavy metals, and 3) investigate synergistic effects of combined phosphorus and heavy metal loadings to determine if such loadings have a greater potential for producing undesirable modifications in the composition and structure of phytoplanktonic communities and enhancing bioaccumulation and dispersal through the food chain. Phytoplankton samples will be collected from Saginaw Bay, a highly eutrophic area, and the western shore of southern Lake Huron, a less eutrophic area of the system. Polyphosphate distributions and abundance in the collected populations will be determined by use of a lead sulfide precipitation procedure which stains polyphosphate bodies for light microscopic examination and by transmission electron microscopy. Laboratory investigations will be conducted with natural phytoplanktonic populations or isolates of algae from the Great Lakes. Polyphosphate formation and degradation will be studied to determine if polyphosphate alters succession by either increasing growth of species containing polyphosphate or by altering assemblage composition through stimulation of other species through release of phosphorus by the polyphosphate containing algae. Similar studies including simultaneous additions of phosphate salts and heavy metals will be conducted to determine if polyphosphate serves as a bioaccumulations pathway for heavy metals and allows subsequent toxicant release upon degradation. Heavy metals will be analyzed in situ by x-ray energy dispersive analysis and scanning transmission electron microscopy. In addition, synergestic effects of combined nutrient and heavy metal loadings will be investigated in microcosms composed of sediments and natural populations from western Lake Huron or Saginaw Bay.

Dr. Sicko-Goad has used her existing research data base to formulate some intriguing questions about the ecological significance of polyphosphates in the phytoplanktonic populations of Lake Huron. Answers to these questions will provide the scientific community with fundamental information on phosphorus kinetics and mechanisms of transport of toxic heavy metals in aquatic systems. Field sampling will be interfaced with the Saginaw Bay Special Field Survey, in which extensive data will be collected on both chemical parameters and phytoplankton species composition and abundance. Data from a previous investigation of Saginaw Bay will be useful in determining if significant biological transport of phosphate from a highly eutrophic area to less affected parts of the ecosystems has occurred and if a 50% reduction in phosphorus loading to Saginaw Bay has resulted in a decrease of stored polyphosphates, or if polyphosphate formation is stimulated by the other factors, such as the presence of trace or heavy metals. The laboratory investigations will provide more specific information on mechanisms of uptake, transport, release/availability of heavy metals by algae. Dr. Sicko-Goad, the Principal Investigator, has extensive experience in electron-microscopy and has used the proposed methods in previous investigations. The Co-Principal Investigator, Dr. E.F. Stoermer, has an extensive background in the taxonomy and ecology of algae. Both investigators are prolific publishers and their past work constitutes an excellent basis for the proposed work. Facilities are readily available for conducting the proposed research and the investigators have used the equipment in previous research projects.

As indicated in her report, Dr. Sicko-Goad has made significant progress toward obtaining her stated objectives. Analysis of field data is underway and effects of phosphate and lead treatments have been noted at the subcellular level. Technical problems have been identified and additional experiments have been planned.

OIL AND RELATED CONTAMINANT EFFECTS ON WATERFOWL IMMUNE DEFENSES:
A PROGRESS REPORT

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ABSTRACT

Experiments have been designed to study the effects of crude oil, petroleum distillates, and oil spill control chemicals on resistance of waterfowl to bacterial or viral infection and their immune responsiveness, both antibody and cell mediated. Toxicity studies have been conducted to determine mallard sublethal oral doses of various contaminants, including South Louisiana crude oil, Bunker C fuel oil, a dispersant-Corexit 9527, and oil/dispersant combinations. Additional assays for measuring immune function in mallard ducks are being developed.

INTRODUCTION

Increased oil consumption with subsequent rises in production and transport has inevitably resulted in the pollution of aquatic ecosystems. Tanker groundings, collisions, blowouts, and ruptured pipelines have spilled oil into oceans, rivers, lakes, inland canals, and estuaries, but these accidents account for only 5% of all oil entering the marine environment (4). Most oil is introduced via discharges from normal transport and refining operations. Along with urban runoff and natural seeps, these intentional discharges have induced persistent or chronic levels of oil pollution near major ports and other areas. It has been estimated that more than 1.5 million metric tons of oil or petroleum products enter U.S. coastal waters each year (4).

The damaging effects of oil on local flora and fauna following major spills have been well documented. Massive dieoffs of animals in the vicinities of oil slicks have received considerable public attention, and oil toxicity studies have been conducted on numerous marine organisms. Much information has been gathered on acute, short term toxicity and carcinogenic properties of oil, however little is known about the sublethal effects of oil, particularly with chronic exposure. Sublethal effects are more subtle than direct mortality and may persist for an extended period of time with long term repercussions.

Efforts to contain oil pollution have often included application of chemical dispersants. Dispersants are intended to remove oil from the water surface, prevent the formation of oil in water emulsions and reduce the ability of oil to adhere to objects. However, chemically dispersed oil may persist longer in the environment, penetrating sand and gravel beaches at a higher rate than non-dispersed oil. Oil spill control chemicals may compound the problems of oil toxicity. The effects of dispersants on wildlife have not been fully investigated. Some combinations of oil and dispersant have been reported to be more damaging than either contaminant alone (1).

Waterfowl have been repeatedly subjected to oil pollution at both acute and chronic levels. High mortality in waterfowl populations after major spills has long concerned ornithologists, hunters, and other wildlife enthusiasts. In general, the effects of oil on waterfowl fall into two categories: physical effects, such as oiled feathers, and systemic effects, which result from the ingestion of oil. Systemic effects can develop from either acute or chronic exposure of birds to oil.

Waterfowl ingest oil by eating contaminated food or drinking water that contain oil in suspension. Laboratory studies have shown that ducks will voluntarily eat food that has been contaminated with petroleum or petroleum products (7,8,9,18). Additionally, observers in the field have noted that birds try to remove oil from their feathers by preening. Ducks will preen up to 50% of the contaminating oil from their feathers during the first eight days after exposure (5). Studies using radioactively labelled crude oil have demonstrated that significant amounts of oil are ingested by contaminated ducks while preening (5). It has been estimated that birds in the wild could encounter and ingest 2-3 gram doses of oil per kilogram of body weight (6).

Most studies of oil ingestion by waterfowl have concentrated on symptoms of overt toxicity and physiological effects. Histological examination of tissues collected from oil contaminated birds reveal irritation of the gastrointestinal mucosa, lipid pneumonia, fatty degeneration of the liver, acinar atrophy of the pancreas, adrenocortical hyperplasia, and toxic nephrosis (6). In a study on the effects of mild cold stress on ducks maintained on petroleum laden diets, adrenal hypertrophy and severe involution of lymphoepithelial tissues were characteristic in all birds that died (9). Particularly interesting were the effects on the thymus, bursa of Fabricius, and spleen weights. In another study on the effects of chronic ingestion of crude oil on mallard ducklings, liver hypertrophy and splenic atrophy were discovered in birds fed 2.5 and 5.0% oil diets (18).

So far no attempts have been made to correlate the observed pathological signs of oil toxicity with altered immunological responsiveness and defense of birds against infectious organisms. Certain polycyclic aromatic hydrocarbons (PAH) are known to suppress the immune response of some animals. Two such hydrocarbons, benzo-a-pyrene (BAP) and derivatives of benz-a-anthracene (BAA), are present at significant levels in many petroleum products (14). Experiments have shown that injection of single low doses of BAP and BAA derivatives into mice prior to administration of an antigen result in depressed levels of antibody forming cells and impairment of graft rejection (16,17). The input of BAP into the marine environment from petroleum spillage has been estimated at 20-30 tons annually (14). Certain metals such as cadmium and lead, which may be closely associated with or derived from constituents of petroleum have also been found to be immunosuppressive (10). Interestingly, an avian cholera outbreak followed a major oil spill in 1978 in the Chesapeake Bay area, resulting in the death of over 10,000 birds (12). Although there was no evidence obtained to relate the two events, the incident bears further investigation.

Our research goal is to both develop and test an avian system for determining the impact of oil and oil related contaminants on host immune defenses, using the mallard duck as a model. The mallard duck (Anas platyrhynchos) is an ideal candidate for oil immunotoxicology studies. Mallards are known to frequent or inhabit coastal areas, marshes, and estuaries that are subject to periodic oil contamination. Postmortem analysis of mallards exposed to petroleum by stomach intubation have shown that petroleum hydrocarbons can accumulate in various tissues (11). Furthermore, mallards are readily available and easily maintained in captivity.

RESEARCH PLANS AND PRELIMINARY RESULTS

Several techniques have recently been developed in our laboratories that permit rapid, economical assessment of alterations in the immune state of mallards. Additional measures of immune function, such as blastogenesis responses to antigens and cell mediated immune responses are currently being adapted to mallards. Toxicity studies have been completed for five contaminants; mallard sublethal oral doses have been established for each. By incorporating the findings of these preliminary studies on mallard immune function and oil toxicity, experiments will be designed to test the hypothesis that oil and related contaminants alter host immune defenses and resistance to infectious disease.

EXPERIMENTAL ANIMALS

All of the experiments in this study are being conducted on adult male mallards purchased from game farms. The ducks are housed individually in cages in the isolation building at Charmany Farms or non-isolated animal rooms at Fred Hall, University of Wisconsin, Madison. A photoperiod of 8 hours light, 16 hours dark is maintained for the duration of every experiment.

ENVIRONMENTAL CONTAMINANTS

Oil samples were obtained from the American Petroleum Institute. For biological effects studies, the institute provides a suite of four reference oils consisting of two representative crude oils and two fuel oils. This study will include South Louisiana crude oil (SLCO), which is an example of a typical major Gulf of Mexico crude oil transported extensively by tankers along the coastal waters of the eastern U.S., and Bunker C fuel oil (BCFO), which is used for powering marine vessels. Corexit 9527, a water soluble, self mixing dispersant, is being tested alone and in combination with both oils. Samples of Corexit have been provided by Exxon.

TOXICITY STUDIES

Twenty-eight day toxicity studies have been completed for five contaminants, SLCO, BCFO, Corexit 9527 (diluted in at least 9 parts of water), SLCO/Corexit and BCFO/Corexit mixtures (50 parts oil to 1 part Corexit). Various amounts of each contaminant were orally administered to adult male mallards daily by stomach tube. Mortality, behavioral abnormalities, and weight changes were monitored. Plasma was collected and stored for future enzyme analysis. At the termination of each experiment, birds were sacrificed for post mortem analysis. Spleen, liver, and kidney weights were recorded. Spleen, liver, kidney, gastrointestinal lymphoid, heart, lung, brain, skeletal muscle, and testis tissues were collected and saved for histological examination.

Our experiments indicate that mallards can ingest a relatively large amount of oil with no apparent signs of illness. In this study, ingestion of up to 15 ml of oil per kilogram of body weight per day was usually not lethal. One out of five birds receiving 15 ml/kg BCFO per day died. None of the birds receiving SLCO died. Some of the birds were able to regurgitate the administered oil, particularly those receiving BCFO. At the low levels, regurgitation was rarely observed. Although most birds lost weight during the course of these experiments, there was no significant difference in weight change between treated birds and controls (Tables 1 and 2). Likewise, mean liver and kidney indices (calculated as ratio of liver or kidney weight to total body weight at time of death x 10,000) did not differ significantly between treatments in either of the oil toxicity experiments (Tables 1 and 2). A decrease in mean spleen weight in birds receiving $\geq$2.5 ml/kg BCFO was the most notable effect of oil ingestion (Tables 1 and 2). Figure 1 depicts the relationship between mean spleen index and dose of BCFO administered. A corresponding decline is illustrated in a plot of mean spleen index versus dose of BCFO (Figure 2). These trends are not the result of decreasing body weight. There was no significant difference in spleen weights between controls and groups treated with SLCO.

TABLE 1. MEAN BODY AND ORGAN WEIGHT INDICES OF MALLARD DUCKS SURVIVING INGESTION OF SOUTH LOUISIANA CRUDE OIL FOR 28 DAYS*

Dose ml/kg	Mean body weight change	Mean liver index†	Mean kidney index†	Mean spleen index†	Deaths
0.0	.06 ± .05	263 ± 41	50 ± 9	7.4 ± 1.2	0/5
1.0	.06 ± .04	248 ± 18	46 ± 5	6.0 ± 1.3	0/5
2.5	.12 ± .08	263 ± 21	54 ± 22	5.8 ± 1.8	0/5
4.0	.09 ± .04	255 ± 36	46 ± 9	6.7 ± 1.8	0/5
7.0	.10 ± .06	269 ± 53	41 ± 7	6.3 ± 1.6	0/5
12.0	.09 ± .02	259 ± 19	47 ± 4	6.5 ± 1.9	0/5

* None of these treatments were significantly different by one-way analysis of variance

† $\frac{\text{Organ Weight (g)}}{\text{Final body weight (g)}}$ x 10,000

TABLE 2. MEAN BODY AND ORGAN WEIGHT INDICES OF MALLARD DUCKS SURVIVING INGESTION OF BUNKER C FUEL OIL FOR 28 DAYS

Dose ml/kg	Mean body weight change†	Mean liver index†	Mean kidney index†	Mean spleen index†	Deaths
0.0	.05 ± .04	250 ± 64	43 ± 6	5.4 ± 1.5	0/4
1.0	.16 ± .11	323 ± 37	49 ± 6	4.3 ± 1.2	0/5
2.5	.20 ± .10	310 ± 26	46 ± 2	3.8 ± 1.3*	1/5§
4.0	.15 ± .17	336 ± 59	44 ± 5	3.3 ± 1.1*	0/5
7.0	.08 ± .12	351 ± 75	46 ± 7	3.5 ± 1.1*	0/5
12.0	.25 ± .11	354 ± 81	43 ± 3	2.9 ± 0.9*	1/5

* Significantly different from controls at $\alpha = .10$

§ Cause of death undetermined - not directly due to oil toxicity

† $\frac{\text{Organ Weight (g)}}{\text{Final body weight (g)}}$ x 10,000

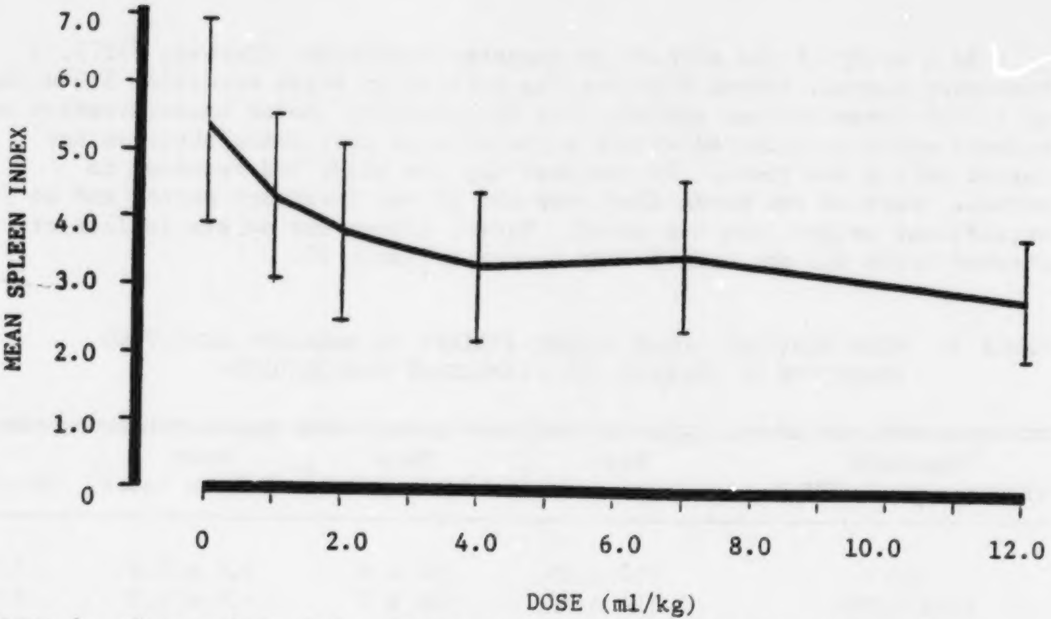

Figure 1. Mean spleen index versus dose of Bunker C fuel oil orally administered to mallards for 28 days.

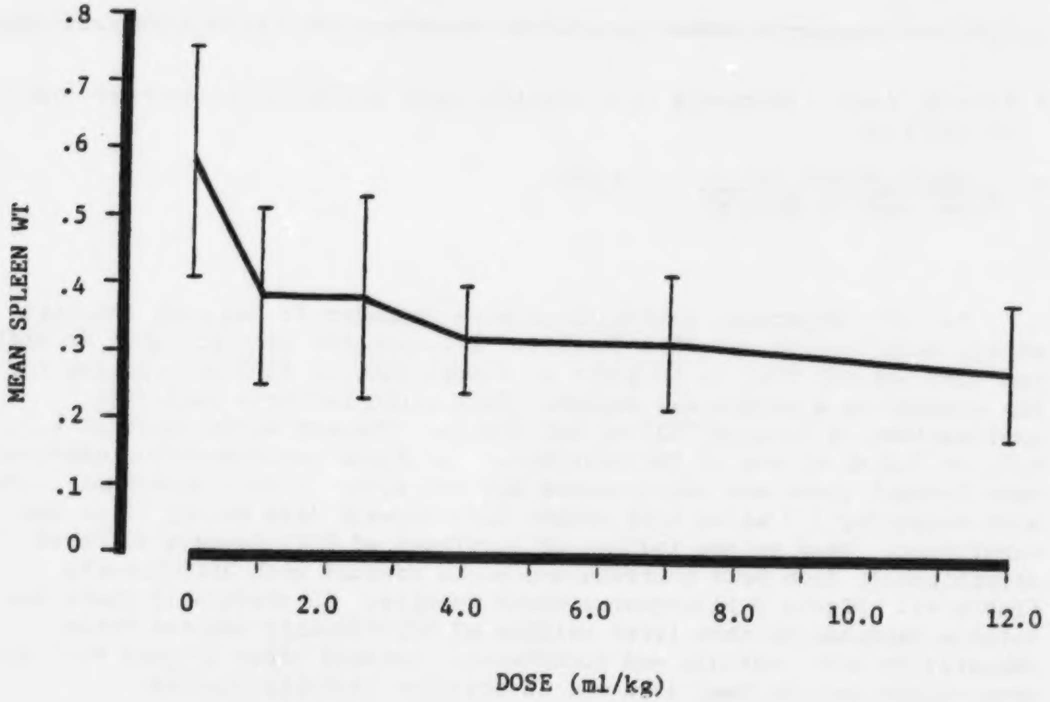

Figure 2. Mean spleen weight versus dose of Bunker C fuel oil orally administered to mallards for 28 days.

In a study of the effects of ingested dispersant (Corexit 9527), a temporary nervous system disorder was noticed in birds receiving 5.0 ml/kg of a 1:10 Corexit/water mixture (0.5 ml Corexit). Motor uncoordination and reduced mobility occurred within a few minutes post administration but lasted only a few hours. By the next day the birds had returned to normal. None of the birds died over the 28 day treatment period and no significant weight loss was noted. Liver, kidney and spleen indices of treated birds did not differ from controls (Table 3).

TABLE 3. MEAN BODY AND ORGAN WEIGHT INDICES OF MALLARDS SURVIVING INGESTION OF COREXIT OIL DISPERSANT FOR 28 DAYS*

Treatment ml Corexit/ml DDH_2O	Mean liver index†	Mean kidney index†	Mean spleen index†	Deaths
0/5	207 ± 29	33 ± 5	3.9 ± 0.8	0/6
.006/4.994	191 ± 22	38 ± 7	4.6 ± 1.3	0/6
.012/4.988	174 ± 49	39 ± 4	4.4 ± 1.3	0/6
.025/4.975	200 ± 9	42 ± 3	4.3 ± 1.5	0/6
.05/4.95	184 ± 25	40 ± 8	3.9 ± 0.9	0/6

* None of these treatments were significantly different by one-way analysis of variance

† $\frac{\text{Organ Weight (g)}}{\text{Final body weight (g)}} \times 10{,}000$

Two oil/dispersant combinations were included in the next toxicity study, SLCO/Corexit and BCFO/Corexit. The mixtures were prepared by adding one part Corexit 9527 to 50 parts of either SLCO or BCFO and shaking for ten minutes on a mechanical shaker. This ratio reflects realistic applications of Corexit 9527 to oil spills. Treated birds received 1.0, 4.0, or 7.0 ml of one of the mixtures. The motor uncoordination observed when Corexit alone was administered did not occur in this experiment. One bird receiving 4.0 ml/kg body weight BCFO/Corexit died on day 24 of the experiment. Mean spleen indices of survivors of BCFO/Corexit differed significantly from both controls and birds treated with SLCO/Corexit (Table 4). Figure 3 illustrates these results. Interestingly there was a notable increase in mean liver weights of BCFO/Corexit treated birds compared to both controls and SLCO/Corexit treated birds (Figure 4). Liver hypertrophy had not been observed in previous toxicity studies.

TABLE 4. MEAN BODY AND ORGAN WEIGHT INDICES OF MALLARD DUCKS SURVIVING INGESTION OF OIL DISPERSANT MIXTURES FOR 28 DAYS*

Treatment	Dose ml/kg	Mean liver index†	Mean kidney index†	Mean spleen index†	Deaths
Control	—	213 ± 31^{a}	40 ± 4^{a}	4.3 ± 1.5^{ab}	0/4
SLCO/C	1.0	277 ± 43^{ab}	45 ± 2^{a}	4.7 ± 0.9^{a}	0/5
SLCO/C	4.0	259 ± 16^{ab}	43 ± 3^{a}	4.5 ± 0.8^{ab}	0/5
SLCO/C	7.0	268 ± 40^{ab}	41 ± 4^{a}	4.4 ± 0.8^{a}	0/5
BCFO/C	1.0	297 ± 53^{b}	43 ± 6^{a}	2.8 ± 0.9^{bc}	0/5
BCFO/C	4.0	395 ± 69^{c}	43 ± 3^{a}	2.8 ± 0.6^{c}	1/5
BCFO/C	7.0	349 ± 45^{c}	44 ± 5^{a}	2.7 ± 0.5^{c}	0/5

* Indices not showing a common letter differ significantly from one another at α = .05

† $\frac{\text{Organ Weight (g)}}{\text{Final body weight (g)}} \times 10{,}000$

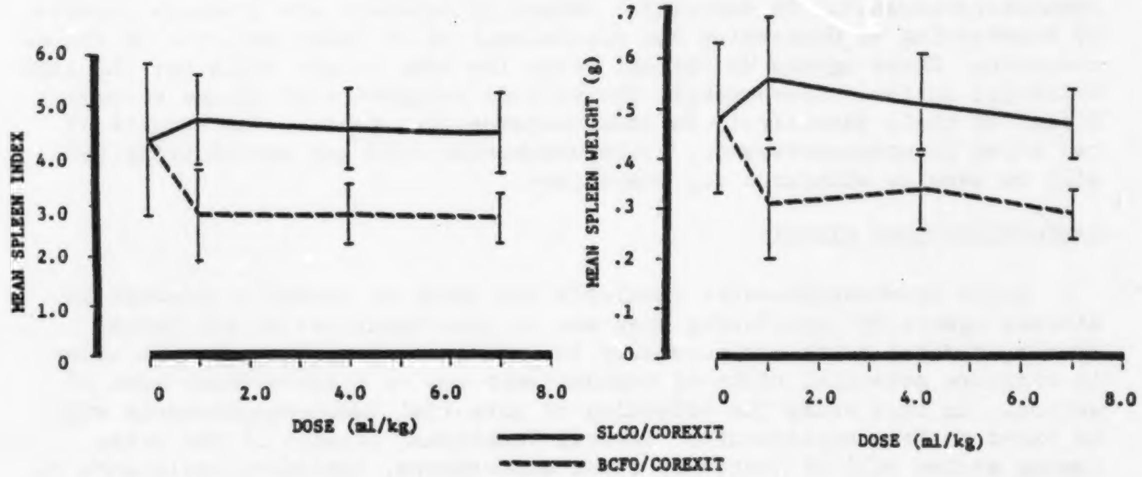

Figure 3. Mean spleen index and weight versus dose of Bunker C fuel oil/Corexit administered to mallards for 28 days.

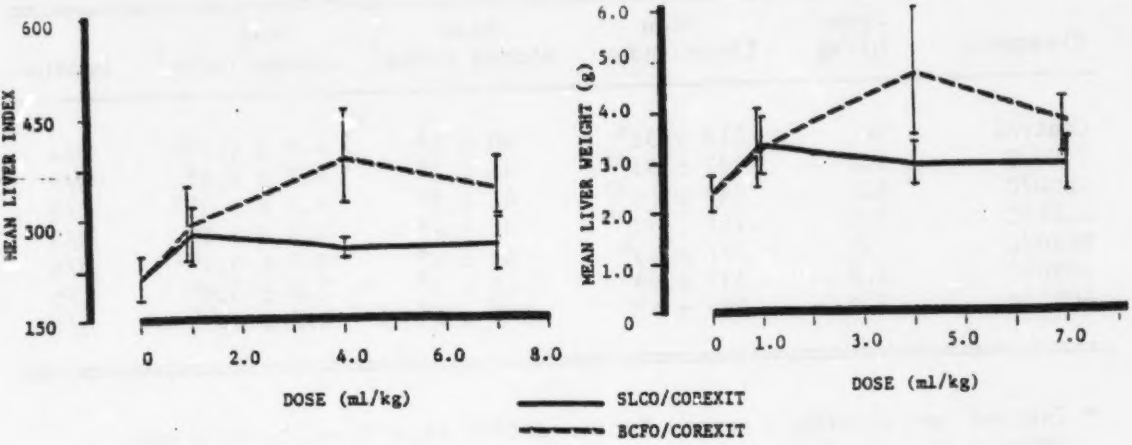

Figure 4. Mean liver index and weight versus dose of Bunker C fuel oil/Corexit administered to mallards for 28 days.

KNOWN IMMUNOSUPPRESSANTS

The effects of oil and oil-related contaminants on host defenses and the avian immune system will be assessed and compared to those of known immunosuppressants. By definition immunosuppressants are products capable of suppressing or depressing the development of at least one type of immune reaction. These agents do not all share the same target cells nor the same cytologic action. Consequently the various categories of immune responses differ in their sensitivity to immunosuppressive agents. The effects of two known immunosuppressants, cyclophosphamide (CY) and azothioprine (AZ) will be used as standards for comparison.

IMMUNOTOXICOLOGY STUDIES

Since immunosuppressive chemicals may alter an animal's response to disease agents by interfering with one or more functions of the immune system, several tests are necessary in immunotoxicologic studies in order to evaluate potential risks of contaminants and to discern their mode of action. In this study the screening of potential immunosuppressants will be based on four experiments. Several functional aspects of the avian immune system will be tested in these experiments, including resistance to infectious disease, antibody mediated immunity, antigen and mitogen induced lymphocyte blastogenesis, and some measure of cell mediated immunity.

1) Protection against infectious agents depends on two components, specific immunity, which includes cell mediated immunity and antibody

mediated immunity, and nonspecific resistance, which is a function of enzymes (such as lysozyme), the interferon system, innate or genetic characteristics, phagocytes, etc. Thus, resistance to challenge with an infectious agent measures overall response. Oil treated mallards will be exposed to two infectious agents - avian cholera, an acute bacterial infection, and duck plague virus (DPV), a disease that can persist in a carrier state in apparently healthy waterfowl.

Avian cholera serotypes of known origin were obtained from the National Fish and Wildlife Health Lab, Madison, Wisconsin. Selection of the appropriate dose and strain of the organism to be used in these experiments is very critical. We are currently conducting challenge experiments on different inoculums in untreated mallards. When the appropriate dose of bacterium has been established, it will be injected into oil treated mallards; percent mortality will be measured.

Several ducks that survived avian cholera infection in our early challenge experiments were included two months later in a pilot study on the toxic effects of ingested oil/dispersant mixtures. After 10 days one mallard receiving approximately 5.0 ml of a 1:30 BCFO/Corexit mixture died. Upon necropsy, typical lesions of avian cholera were noted, including an enlarged spleen and a haemorrhagic heart. *Pasteurella multocida* was subsequently isolated from the spleen. Although no conclusions can be drawn from this isolated observation, the circumstances suggest that the BCFO/Corexit intoxication may have altered host resistance to some extent, possibly causing an abrupt upwards shift in the level of *P. multocida* infection resulting in septicemic death of the bird.

2) Duck plague virus will be used to assess the effects of oil and oil-related compounds on persistent infections. Carrier birds, defined on the basis of oral erosions under the tongue and/or shedding of virus in the feces, will be given oral doses of each contaminant. The amount and frequency of DPV shedding will be determined over the 28 day treatment period and two weeks thereafter, and compared to controls. DPV shedding will be determined by inoculation of duck embryo fibroblastic cell cultures with eluates from periodic oral and cloacal swabs. The cells will be tested for DPV by our standard direct fluorescent antibody test. Shedding of virus in feces will be evaluated in terms of altered immune response.

In vitro assays of lymphocyte proliferation upon stimulation with mitogens, such as phytohemagglutinin (PHA) or pokeweed mitogen (PWM), have been extensively used to index lymphocyte activity. Lymphocyte stimulation assays with various mitogens have been applied to mallards by Drs. Sauch and Hinsdill. A similar but more specific assay is antigen-induced lymphocyte immunostimulation (13). This assay is currently being developed with an antigenic preparation of DPV vaccine. In this study, the compounds under test will be administered to DPV carrier mallards. Peripheral blood lymphocytes will be collected before and after contamination for immunostimulation assays with inactivated DPV vaccine.

3) The production of antibodies to a specific antigen requires the

action of several subpopulations of cells, including B cells and T helper cells. In this study of general immune responsiveness, antibody mediated immunity is measured by counting spleen plaque forming cells. For this procedure, mallards are immunized with sheep red blood cells (SRBC). Four days after the initial immunization, birds are sacrificed to remove their spleens. Direct spleen plaque forming cell assays are performed according to methods developed by Cunningham and Szenberg (3) that have been adapted for mallards by Drs. Sauch and Hinsdill.

A preliminary study testing the effects of oil pollutants on antibody mediated immunity was recently completed. Groups of mallards were treated with either SLCO, BCFO, or Corexit for 28 days. On day 29 birds were inoculated intravenously with SRBC. A known immunosuppressant, cyclophosphamide (CY), was orally administered to another group two times prior to SRBC inoculation. Four days after the injection the birds were sacrificed. Mean liver, kidney and spleen weights are recorded in Table 5. As before, mean spleen weight of birds receiving BCFO is lower than the control value. Mean spleen weight of CY treated birds is similarly reduced. Figure 5 illusrates this comparison.

TABLE 5. MEAN ORGAN WEIGHT INDICES OF MALLARD DUCKS AFTER INGESTING OIL, DISPERSANT, OR CYCLOPHOSPHAMIDE*

Treatment	Dose ml/kg	Mean liver index†	Mean kidney index†	Mean spleen index†
Control	—	169 ± 25[a]	32 ± 4[a]	5.7 ± 1.0[ab]
Corexit (1:10)	5.0	164 ± 18[a]	33 ± 4[a]	6.0 ± 1.2[ab]
SLCO	1.0	178 ± 22[a]	32 ± 6[a]	5.5 ± 0.8[ab]
SLCO	5.0	187 ± 22[a]	34 ± 6[a]	6.2 ± 2.0[a]
BCFO	1.0	199 ± 26[a]	34 ± 3[a]	4.8 ± 0.6[bc]
BCFO	4.0	172 ± 31[a]	33 ± 4[a]	4.5 ± 1.1[c]
CY	50.0	163 ± 36[a]	28 ± 4[a]	4.4 ± 1.4[c]

* Indices not showing a common letter differ significantly from one another at $\alpha = .05$.

† $\frac{\text{Organ Weight (g)}}{\text{Final body weight (g)}} \times 10{,}000$

The number of plaque forming cells (PFC's) to SRBC in the spleen of a sensitized animal is an indication of the antibody forming capability of that animal. The number of PFC's are standardized between animals by counting the number of viable cells per gram of spleen. In Figure 6, which illustrates data collected from this preliminary study, mean PFC's per 10^6 viable cells are expressed as a percentage of the mean control value.

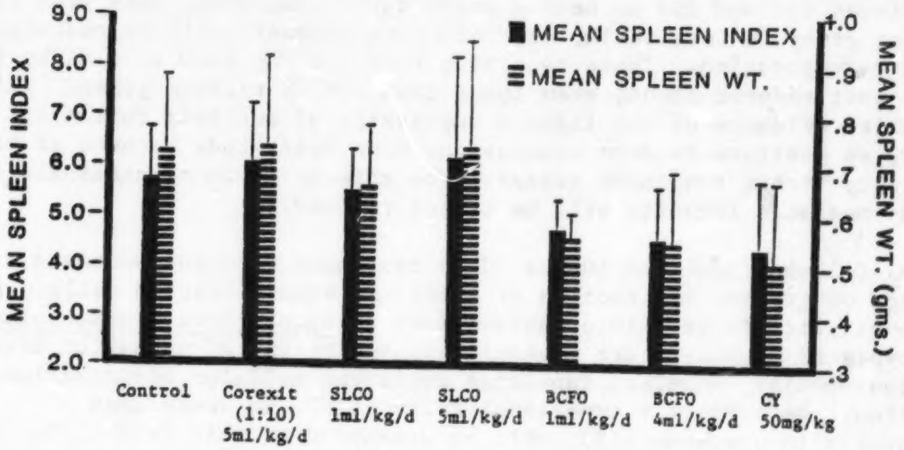

Figure 5. Mean spleen index and spleen weight versus toxicant administered to mallards.

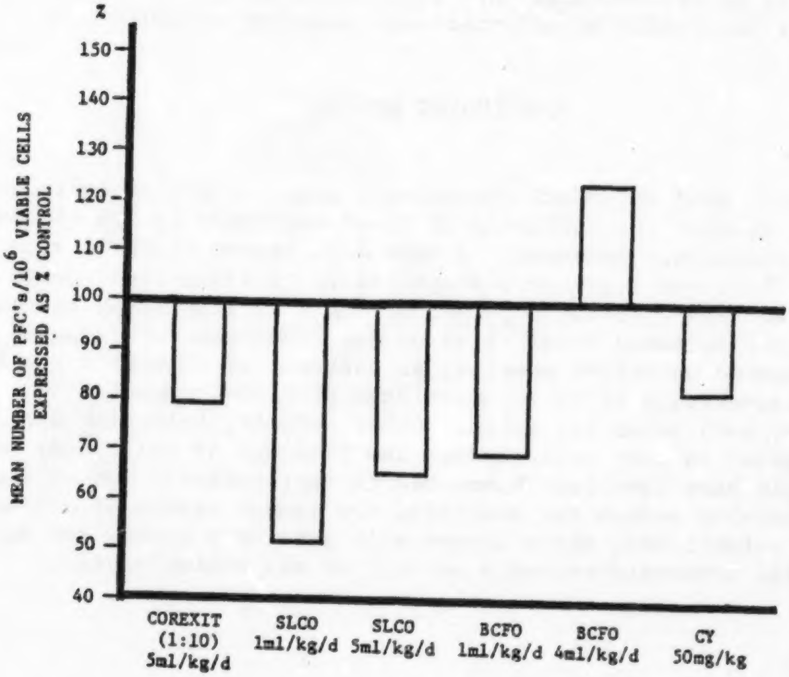

Figure 6. Mean number of PFC's per 10^6 viable cells expressed as percent control versus toxicant administered to mallards.

Those receiving 4 ml/kg BCFO had the highest mean PFC value. Unfortunately, many of the birds in this group were able to regurgitate the administered oil and did so nearly every day. Therefore, data from this treatment group is unreliable, and future experiments will be redesigned to prevent regurgitation. Those receiving 1 or 5 ml/kg SLCO or 1 ml/kg BCFO had the most reduced count; even lower than the CY treated group. This is presumptive evidence of oil induced supression of antibody formation. However, we hesitate to draw conclusions from this study because of high variability within treatment groups. The effects of these chemicals on antibody mediated immunity will be tested further.

4) Cellular mediated immune (CMI) responses include phenomena such as rejection of grafts, destruction of tumor and virus-infected cells, and hypersensitivity to certain organisms such as Mycobacterium tuberculosis. These types of responses are commonly assayed in inbred mammalian hosts; for avian species, however, knowledge regarding cellular mediated immunity is limited. Delayed type hypersensitivity (DTH), an assay used successfully in chickens (15), will be attempted in this study. Mallard ducks sensitized to killed strains of M. avium by intraperitoneal injection will be tested for the DTH response by injection of avian purified protein derivative (PPD) into wing webs, foot webs, or the mucocutaneous junction of the cloaca. The site which yields the most consistent DTH response and the appropriate time of PPD injection will be determined. If feasible, this assay will be incorporated into later experiments for assessing the effects of oil pollutants on cell-mediated immunity of ducks.

CONCLUDING REMARKS

It is known that waterfowl encounter a wide variety of environmental contaminants, however the influence of these compounds on the immune system has not been completely assessed. A depressed immune state in a densely concentrated flock may increase susceptibility to infectious agents. Epidemics in certain wildlife populations could substantially reduce recreational and economic benefits of an important natural resource. For certain endangered waterfowl species, an outbreak of disease might be devastating, especially if it is associated with environmental contamination, such as an oil spill. Other animals, including man, are similarly exposed to many pollutants. The findings of this study on waterfowl might have important human health implications. We will continue our work to develop assays for assessing the immune status of mallard ducks. Once established, these assays will provide a system for screening other potential immunosuppressants as well as oil contaminants.

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Comments on Oil and Related Contaminant Effects on Waterfowl Immune Defenses

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The objectives of this project are to measure the resistance of mallard ducks to bacterial or viral infections and their immune response after pretreatment with crude oil, petroleum distillates, and selected chemicals dispersants.

The importance of this project stems from the fact that offshore drilling operations and oil transport via marine vessels have intensified in recent decades, siginificantly increasing the input of oil into aquatic ecosystems. Accidental spills derived from these coupled with bilge washing, runoff from land based operations, ruptured pipelines, and natural seeps have intensified oil pollution near major ports and other areas.

In order to control oil spills, chemical dispersants are often applied to enhance the removal of oil from water surface and from objects. As a result, the dispersed oil may be more persistent in the environment by penetrating beach materials at a higher rate than nondispersed oil.

Massive dieoffs of animals following major oil spills have been well documented, but little is known of the sublethal effects of oil and particularly the effects of chronic exposure. Furthermore, less is known of the effects of oil dispersants and the detergents used to clean animals exposed to oil. Combinations of oil and dispersant may compound the problem. Most animal recovery efforts have been unsuccessful, especially when attempting to rescue and rehabilitate waterfowl by cleaning oil with the aid of detergents and solvents. This project does not deal with the direct physical or external effects of oil or chemicals, but rather the systemic effects which result from ingestion of oil and/or dispersants.

Many studies have detailed the symptoms of overt toxicity and physiological effects of ingestion of oil by waterfowl but no attempts have been made to correlate these findings with alterations in immune responses and defense of birds against infections organisms. Since other environmental contaminants have been shown to alter immune function in a variety of animals, compounds in oil distillates, dispersants, and detergents may also have effects on host defenses. At least one significant avian cholera outbreak followed a major oil spill in 1978 resulting in massive mortality to birds. There was no evidence obtained to relate the two events but such rouses suspicion and support for investigation of such an incident.

The Environmental Protection Agency's (EPA's) Environmental Biology Review Panel recommended approval of this project because it would be beneficial to determine if oil and the chemicals used to clean up birds and the environment could substantially alter an animal's resistance to infections disease. If such effects are found, this research will provide a system for screening other potential environmental hazards to waterfowl. The use of dispersants or detergents that interfere with disease resistance should then be prohibited.

Waterfowl are literally under the gun from a variety of angles, including pollution effects. Corrective measures are being employed in some instances, e.g. steel shot for hunting in place of lead in the case of heavy metal effects. Here is a chance of finding solutions to another possible chronic toxicity problem.

Lastly, I find this an interesting new avenue of investigation for EPA. This is an area (immuno-suppression) typically housed in other agencies such as the National Science Foundation or the National Institutes of Health. I think it refreshing that we delve into the development of this technology and make use of the expertise of Dr. Yuill's group.